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Faculdade de Medicina Veterinária

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DISSERTAÇÃO DE MESTRADO INTEGRADO EM MEDICINA VETERINÁRIA

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To Towa & Jinsei.

ACKNOWLEDGEMENTS

First and foremost, thank you to my thesis coordinator and mentor, Professor Ana Mafalda. It has been a long road and I am grateful for all the support and belief which you instilled in me. Today I am above all, happy to have gained a friend for life.

Thank you to the orientation of Professor Berta São Braz, without whose dedication and support this thesis could not have been orchestrated in the manner that it was.

To the dermatology dream team – Thank you Prof. Mafalda, Lúcia and Joana, for the time and care dedicated to helping me with the patients in this study, for making hard work fun and for all the laughs and 5-minute "lunch" breaks by the vending machine on 12-hour days filled with overbookings.

To the colleagues at the FMV Hospital - thank you for taking the time to help in recruiting the patients for this study.

To Professor Telmo Nunes – Thank you for having all the patience in the world to teach me the language of statistics and for being a life coach with words of wisdom and honesty.

To the owners of the dogs included in this study – thank you for giving me a chance.

To Professor Maria Constança Pomba and her laboratory for establishing the invaluable link between clinical and laboratory results, in particular to Eng. Adriana Belas – Thank you for undertaking the essential work in this project.

To Dr. Isabel Pimenta (Biolotus®) – You believed in this project when it was just a seed waiting to be planted and there was nothing yet to show. Thank you.

To João Miguéns (Biolotus®) – Thank you for the support and hard work in providing material.

To Mr. Joost Postmes (Triticum®) – Thank you for giving us this opportunity and for allowing us to spread the word regarding honey. One step at a time, the world will get there.

To Mariana – Your last minute help was invaluable and I know you know I couldn´t have done it without you! Thank you!

To Cristina Tinkerbell – Thank you for your friendship, advice and support. Knowing I could count on you along this journey made the hard times easier to bear.

To Carlita – Your good disposition always brought a smile to my face and made cloudy days brighter.

Mom and Dad – I was never easy but you didn´t give up on me and words are not nearly enough to express my gratitude towards all the love you gave and the sacrifices you made for me to be here. I´m glad I have the rest of my life to keep thanking you. If one day I can be even half the parents you were to me, I will be content.

Jin – The little brother I´ve always looked up to. Thank you for putting up with me for all these years even though you never had a choice. I hope I´ve made you proud.
Bu – The definition of unconditional love. You got me through everything. Thank you.
To those of you who go unnamed - Thank you for coming into my life. May you always stay.

Thank you to Triticum® (Maastricht, Netherlands) and Biolotus® (Lisbon, Portugal) for kindly providing the materials and funding necessary for this study.

ABSTRACT

"L-MESITRAN® IN THE MANAGEMENT OF CANINE OTITIS EXTERNA – A PILOT STUDY"

At a time when antimicrobial resistance is rising steadily and the involved microorganisms are demonstrating zoonotic potential, honey and its derived products may prove useful in this ongoing battle. Otitis externa in dogs is considered to be one of the most prominent causes for presentation at veterinary practice. Some of the regularly administered agents in otitis treatments are no longer effective, as resistance has increased, perhaps due to the often long-term periods which are necessary for resolution and the accompanying tendency towards chronicity. In order to address the need for efficient alternative treatments, L-Mesitran® Soft, a medical grade honey gel was used to treat 15 dogs with otitis externa of bacterial and/or fungal involvement. Success was based on clinical score decrease, cytology and owner input over time and with basis on culture results. 70% of enrolled dogs achieved clinical cure between days 7 to 14 and over 90% on day 21, the maximum established time limit, with a confidence interval of 95%. Furthermore, by day 7, 20% of dogs had obtained both clinical and cytological cures. This study was successfully able to demonstrate that the use of L-Mesitran® was effective in managing otitis externa in dogs, including cases in which highly resistant pathogens were present, such as methicillin-resistant *Staphylococcus pseudintermedius* (MRSP), thus paving the way to future studies.

Key-words: honey; antimicrobial resistance; canine; otitis externa; L-Mesitran® Soft; methicillin-resistant *Staphylococcus pseudintermedius* (MRSP).

RESUMO

"L-MESITRAN® NO MANEIO DE OTITE EXTERNA CANINA – UM ESTUDO PILOTO"

Numa altura em que nos deparamos com um aumento de bactérias com resistências aos antibióticos e em que os organismos envolvidos apresentam por vezes inclusivamente potencial zoonótico, o recurso ao mel e seus derivados pode ser uma inestimável ferramenta no decurso desta batalha. A otite externa em cães é um estímulo iatrotrópico frequente e das principais causas de idas ao médico veterinário. Alguns dos tratamentos habitualmente utilizados deixaram de ser eficazes à medida que as resistências surgiram, talvez consequência de terapêuticas prolongadas e recorrentes. Neste estudo avaliou-se uma potencial alternativa à terapêutica, recorrendo-se ao L-Mesitran® Soft, uma pomada contendo mel de grau clínico, para tratar 15 cães com otite externa de envolvimento bacteriano e/ou fúngico. A resposta foi considerada positiva de acordo com a diminuição da pontuação clínica, citologia e opinião dos donos, no decorrer do tempo. Estabelecido o limite de 21 dias, 70% dos cães tratados obteve cura clínica entre os dias 7 e 14 e mais de 90% no dia 21, com um intervalo de confiança de 95%. Ainda, até ao dia 7, 20% dos cães havia obtido cura clínica e citológica. Este estudo demonstrou que o L-Mesitran® foi eficaz no maneio dos casos de otite externa, incluindo aqueles em que estavam presentes bactérias com várias resistências aos antibióticos, como é o caso do *Staphylococcus pseudintermedius* com resistência à meticilina (SPRM), abrindo assim caminho para futuros estudos.

Palavras-chave: mel; resistência antimicrobiana; canina; otite externa; L-Mesitran® Soft; *Staphylococcus pseudintermedius* resistente à meticilina (SPRM).

GENERAL INDEX

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

CAD	Canine Atopic Dermatitis
CADESI-04	Canine Atopic Dermatitis Extent and Severity Index-04
CE	European Conformity
CFU	Colony-forming Units
ESBL	Extended-spectrum Beta-lactamase
FDA	Federal Drug Administration
H_2O_2	Hydrogen peroxide
IL	Interleukin
LAB	Lactic acid bacteria
LL/2	Lewis Lung Carcinoma/2
MBC	Minimum Bactericidal Concentration
MIC	Minimum Inhibitory Concentration
MM6	MonoMac-6
MRSA	Methicillin-resistant *Staphylococcus aureus*
MRSP	Methicillin-resistant *Staphylococcus pseudintermedius*
OTIS3	0-3 Otitis Index Score
PAI	Plasminogen activator inhibitor
PBS	Phosphate buffered saline
ROS	Reactive oxygen species
SPF	Specific-pathogen-free
TNF-α	Tumor necrosis factor-α
VAS	Visual analog scale
VRE	Vancomycin-resistant enterococci

I. INTRODUCTION

1. REVIEW OF CURRICULAR INTERNSHIP – WEST CROSS VETERINARY HOSPITAL, JAPAN

The initial half of the mandatory curricular internship took place at West Cross Veterinary Hospital in Tokyo, Japan, under the supervision of owner and head veterinarian, Dr. Nobuyori Tsukagoshi. This location was chosen for various reasons ranging from the curiosity of how veterinary practice differs on the other side of the world to the desire of experiencing living as a local resident in Tokyo. Seeing as language was going to be an enormous and possibly impeditive barrier, West Cross Veterinary Hospital was the appropriate choice due to it´s bilingual environment and Dr. Tsukagoshi´s veterinary background at an American university. While the use of Japanese language was a pre-requisite for interning there, fluency was not. English was used constantly and required when attending to foreign owners who did not speak Japanese either. West Cross´s location was also highly appealing as it tended to the needs of residential areas known for its many foreigners and a community of people who cared dearly about and were able to invest in treatment for their pets.

During over 500 hours I was able to participate actively in the clinic´s activities, which ranged from general clinical practice to general surgery. When consultations were in Japanese I observed firstly and paid attention to the anamnesis and any questions I had regarding the case were addressed in private to Dr. Tsukagoshi. If the owners happened to be from a country other than Japan I would begin the consultation and then communicate the information to my supervisor. This interaction with the foreign owners was a very positive experience in the sense that I was given more responsibility towards them and the bond was stronger due to the common language. With regard to practical activities in this sense I was able to review how to perform basic auxiliary diagnostic procedures such as Diff-Quick staining for cytology and use of technical equipment for biochemistry analysis and urine and blood processing.

One of the most important aspects of the practical component at West Cross was the large emphasis on ultrasound diagnostic skills. The clinic had the convenience of possessing its own ultrasound machine for immediate diagnostic aid during the consultation itself and this allowed for much learning and hands-on experience. Basic normal anatomy was the first area that we focused on and from there we approached pathological situations. This constant practicing was extremely useful, as I personally felt that diagnostic imaging had always been one of the most challenging to me. Towards the end of my internship I was encouraged to

make my own diagnosis and transmit it to the rest of the staff, after which one of the attending veterinarians would confirm it. There were various scenarios in which I was instructed to communicate the diagnosis to the owners and perform the ultrasound exam in their presence whilst explaining to them what appeared on the screen during the exam. Once I had become more accustomed with medical and anatomical terminology in Japanese I began to do the same with the local pet owners. The same kind of exercise was applied to echocardiography, though with slightly less frequency.

2. REVIEW OF CURRICULAR INTERNSHIP – FACULTY OF VETERINARY MEDICINE, UNIVERSITY OF LISBON, PORTUGAL

The second half of the internship focused entirely on the area of clinical dermatology and immuno-allergology, under the close mentoring of Professor Ana Mafalda Martins, at the FMV teaching hospital. During this time I accompanied the specialty consultations and after a short period of observation commenced with obtaining patients´ anamnesis. This process allowed for an understanding of background context in order to develop a comprehensive rationale of the cases at hand but also provided an invaluable opportunity to exercise clinician to owner communication, a component that is crucial to clinical practice but oftentimes overlooked.

With regard to practical procedures I was taught all of the steps necessary to conduct a complete dermatological evaluation. Skin scrapings, both superficial and deep, were made in order to assess for presence of mites and combing of the hair coat for fleas or ticks, for example. Evaluation of the hair condition was done through hair plucking and such information provided evidence ranging from self-inflicted trauma to infection to parasitic infection to genetic conditions of the hair coat.

Another interesting aspect of the internship in dermatology and immuno-allergology was the observation of allergic reactions through intradermal skin tests. These were also an example of dermatology´s many diagnostic procedures which allow for immediate conclusions and answers.

One of the most valuable assets that I will take from this half of the internship is undoubtedly the value of microscope as a crucial tool in aid and confirmation of dermatological diagnosis. Learning to collect samples and stain them in order to be observed and interpreted was essential and many times provided the convenience of being able to make an immediate diagnosis and if not, an exclusion of one. Identifying common microorganisms from cocci to rods to yeasts and learning to interpret infection or absence of it by quantifying them helped

the process of evolution as a future clinician and could only be perfected through constant practice. On some occasions biopsy samples were required and I was taught the correct procedures for such, many times with use of a biopsy punch.

Seeing as the subject of this thesis pertains to otitis externa a large portion of my time at the hospital was concentrated on the ears and the final stages of the internship were dedicated entirely to patients with ear disease. Knowledge of basic routine otoscopy was acquired throughout consultations and the goal of visualizing intact tympanic membrane or absence of it was accomplished. Auricular cytologies were also performed and stained for viewing under the microscope and where it was deemed necessary samples were also collected for microbial culture and sent to the in-house laboratory. For cases in which the tympanic membrane could not be observed with use of a simple otoscope or in instances of doubt I was taught to make use of a video-otoscope. Such device allowed for direct and clear observation of the entire ear canal and permitted a very detailed evaluation of the internal condition of the ear. When animals presented with excessive and/or solidified cerumen, thus impeding visualization of the canal, a lavage was necessary so as to permit the examination. The video-otoscopic examinations allowed for familiarization with the ear´s anatomy, one which is quite elaborate and fragile. In cases of suspected or confirmed presence of foreign bodies within the ear, removal procedures were conducted. These instances called for careful and precise manipulation of fragile equipment in an even greater fragile environment, that of the ear.

Perhaps the most rewarding aspect of this internship was the opportunity to observe patient progression and the privilege of establishing relationships with owners and their companion animals. My previous notion of clinical practice was that of numerous different patients and cases, most of which would be seen once by a clinician and then perhaps seen by another during a follow-up, if a follow-up were called for at all. The great difference between specialty consultations, in this case, dermatology, is that one can accompany the evolution and monitor firsthand the results and constantly receive owner feedback, making practice even more rewarding on a personal level. I believe that there is great value in creating trust between owners and veterinarians and that communication and listening skills are key to maintaining owner loyalty and through being present at these consultations with Professor Ana Mafalda I was able to determine what kind of a clinician I myself would like to be.

II. REVIEW OF LITERATURE

1. HONEY

1.1. Honey throughout history

*"*There comes forth from their bellies, a drink in varying colour wherein is healing for men*"* (Quran 16:69, Mohsin Khan). Before the world knew sugar, there was honey. Since pre-historic times it has been depicted as a part of human life, as numerous pieces of artwork dating from the Stone Age indicate. The earliest evidence is a cave painting of over 10000 years in eastern Spain, which shows the arduous quest by man to collect honey from a beehive and Ancient Egyptian hieroglyphs hint at the domestication of bees through reference to clay hives (McGee, 2004).

The first written reference to honey interestingly refers to its medicinal use and lies in a Sumerian tablet from 2100-2000 BC, in which there is mention of it as a drug and ointment (Mandal & Mandal, 2011). A number of Egyptian papyruses also later made reference to honey as being prescribed for external use in various conditions, post-operatory treatments, as an anti-inflammatory and even as a suppository (Bogdanov, 2014).

Though written reference to honey refers to its healing properties it is clear that throughout history many civilizations regarded it not only as an important food source but also as a symbol in religion and ceremonies. Hajar (2002) outlines some of the most interesting historical uses for honey among different civilizations. In Greek mythology the almighty Zeus avoided being eaten by his father thanks to the bee-nymph Melisseas, who fed him the honey, which made him strong enough to seize the throne. It is said that Cleopatra´s cosmetics were honey-based and other women from Arabia valued its softening properties and applied it as a facial mask. Pharaohs utilized honey in wedding celebrations, during which the newlyweds would drink honey for good luck and happiness, such that the term honeymoon originated from this time and was then passed on to Greco-Roman culture, still used to this day. Honey was a common offering to the gods in Ancient Egypt and the dead of nobility were buried in or with jars of honey. Tutankhamen´s tomb was found to enclose vast quantities of honey in jars and it is said that Alexander the Great himself was buried in honey (Hajar, 2002).

1.2. Honey production

The Codex Standard for Honey (1981) describes it as the sweet substance produced by honeybees from various plant nectars or by collecting and transforming the excretions of insects that live by sucking portions of plants. Molan (2012a) states that honey is mostly produced by the honeybees from the nectar obtained from different flowers, yet they may also collect the phloem sap of plants in the form of honeydew, which drips after activity by aphids.

Honeybees (*Apis mellifera*) are extremely advanced insects that live in a complex organizational structure, similar to that of a society. A bee colony consists of various members assigned to a number of different cargos entailing specific duties, all of which ultimately answer to one large queen. With tasks ranging from food collection to habitat defense to communication, bees work in a methodically orchestrated manner, much more evolved than that of solitary insects (Mid-Atlantic Apiculture Research and Extension Consortium [MAAREC], 2014). Forager bees, for example, are tasked with collecting the nectar from flowering plants by drinking it and storing it within their crop, or honey stomach, though no digestion occurs there. These such bees then take the nectar back to their hive and regurgitate it directly into the crop of a processor bee, after which they return to the flowers to repeat the cycle. The processor bees then regurgitate the nectar into hexagonal wax cells within the honeycomb for ripening (Shipman, 2013).

The ripening process is a collective one and involves enzyme secretion on behalf of the honeybees every time regurgitation occurs, particularly invertase, which promotes the breakdown of sucrose into glucose and fructose. The nectar is composed largely by sucrose and water. Next the bees remove the water content from the nectar, or dry it, by fanning their wings to create airflow around the honeycomb and aid in its evaporation (Shipman, 2013). In the end this process forms a thick syrup that remains sealed in the hexagonal cells of the honeycomb (Molan, 2012a).

1.3 General properties of honey

"Honey is a natural sweetener, but it is not just a sweetener it´s nature´s gift to mankind" (Singh et al., 2012, p. 12). Honey, in its essence, is a supersaturated viscous solution with a carbohydrate content of 80-85%, most of which is integrated by sucrose, glucose and fructose (Buba, Gigado & Shugaba, 2013; Molan, 2012a). Nearly all of the sucrose is changed into glucose and fructose, which in the end account for up to 90% of honey´s total sugar content (Molan, 2012a).

Before advances in research were made regarding the precise composition of honey it was believed that the monosaccharides glucose and fructose and the disaccharide sucrose integrated it entirely. However, with the evolution in techniques for separation and analysis of sugars, 22 other more complex sugars were found to be present in honey, although glucose and fructose account for the vast composition (Matej, 2004). Such complex sugars end up accounting for 10% of the total sugar content of honey (Molan, 2012a). Curiously, most of these sugars are not found directly in the nectar but are results of the enzymes generated by

honeybee activity, especially invertase, during the ripening of the honey or through chemical action of the acid-sugar mixture in the honey itself (Matej, 2004; White & Doner, 1980).

1.3.1. Enzymes of honey

There are numerous enzymes present in honey, all of which contribute to its functional properties, making it a unique sweetener when in comparison to others, with the most predominant being invertase, diastase and glucose oxidase (Ropa, 2013). The honeybee secretes invertase from its salivary glands and into the honey sac, where the enzyme hydrolyzes the breakdown of sucrose into glucose and fructose, in other words, inverts sugar (Matej, 2004; Ropa, 2013). This enzyme also catalyzes the synthesis of more complex carbohydrates, as it integrates a slightly reversible reaction. When invertase is present in processed or sealed honey it continues to promote the breakdown of sucrose to ripen and mature whilst in storage (Matej, 2004).

Diastase digests starch to simple compounds such as maltose and is also added to the nectar by the honeybees during the collection and ripening processes of honey (Buba et al. 2013; Ropa, 2013). This enzyme´s function is unknown seeing as no starch is present in honey but it has been used as an indicator of quality in European countries, presumably due to its varying levels in different types of honey and its ability to be measured. Despite this common practice, diastase levels do not correlate with honey quality, as its levels can be affected by numerous factors such as floral origin, bee foraging patterns, pH variations and long storage conditions with varying temperatures (Ropa, 2013).

Gluxose oxidase is secreted from the honeybees´ hypopharyngeal gland and into the nectar to aid in honey formation. It catalyzes the conversion of glucose to gluconolactone, which then yields gluconic acid, the principal acid in honey and hydrogen peroxide, which greatly accounts for honey´s antibacterial effect (Matej, 2004; Ropa, 2013). The slightly acidic pH of honey is attributed to this and to other organic acids and is responsible for differences in taste among various types of honey (Matej, 2004).

The enzyme catalase, on the other hand, works in an opposite manner to that of glucose oxidase in that it hydrolyzes hydrogen peroxide to oxygen and water (Brudzynski, Abubaker, St-Martin & Castle, 2011). While the latter enzyme is formed by bees and thus depends on the age and health of the foragers (Pernal & Currie, 2000, as cited in Brudzynski et al., 2011, p. 1) as well as the quality and nature of their diet (Alaux et al., 2010, as cited in Brudzynski et al., 2011, p. 1), the former is originated from flower pollen (Brudzynski et al., 2011; Weston, 2000). The levels of hydrogen peroxide in a given type of honey are therefore determined by its respective levels of the enzymes glucose oxidase and catalase (Weston, 2000).

1.3.2. Other components of honey

In addition to its primary composition of sugar and water, honey also contains numerous other substances such as mineral and nitrogenous compounds to vitamins and trace elements of nutrition (Eteraf-Oskouei & Najafi, 2013; Molan, 2012a). Mineral compound concentration ranges anywhere between 0.1% to 1.0%, with potassium as the major component, followed by calcium, magnesium, sodium, sulphur and phosphorus (Eteraf-Oskouei & Najafi, 2013). Nevertheless, with regard to edible honey, the quantities of such compounds are much too low to be considered of any nutritional value in relation to the recommended daily intake. Besides these elements, honey also contains diverse polyphenols, such as flavonoids, which possess significant antioxidant activity, possibly also contributing to honey´s healing properties (Molan, 2012a).

1.4. Healing properties of honey

The clinical application of honey was abandoned in modern Western medicine due to the discovery and rise of antibiotics, becoming essentially limited to traditional medicine in certain cultures. Currently, the incomplete knowledge regarding the antibacterial characteristics of honey in combination with variability among activity pose large obstacles for the return of its applicability in modern medicine (Kwakman & Zaat, 2012). " (…) the time has now come for conventional medicine to lift the blinds off this ´traditional remedy´ and give its due recognition" (Zumla & Lulat, 1989, p. 385).

The antibacterial activity of honey was initially attributed to its high sugar content, with the consequent osmotic process thought to be responsible for disrupting bacterial cells by drawing out their water content (Molan, 2012b). In 1892, Dutch scientist Van Ketel was able to demonstrate honey´s bactericidal activity (as cited in Dustmann, 1979). In 1919 a study by Sackett would also contradict the previous belief that sugar was responsible for the major activity with a surprising result, through the observation that the antibacterial potential of honey in fact increased through the dilution of honey with water. Years later, Dold, Du & Dziao (1937) revealed the discovery of an antibacterial factor which they named "inhibine" (as cited in Molan, 2012b, p. 2). This term was utilized until 1963, when White, Subers & Schepartz showed through their studies, that inhibine was, in fact, hydrogen peroxide (H_2O_2).

1.4.1. Sugar content

Approximately 80% of honey consists of sugars, mainly glucose and fructose, with usually less than 18% water composition (Kwakman & Zaat, 2012; Molan, 2012a), such that the osmolarity is enough on its own to inhibit growth of certain bacteria and fungi (Molan, 2012b). The coupling of high sugar concentration with extremely low moisture promotes

osmotic stress that also prevents the spoilage of honey by microorganisms (Kwakman & Zaat, 2012). However, high water content that may promote excessive dilution of honey can compromise the antibacterial activity. Bacteria are much more susceptible to high sugar concentrations than are fungi, which will grow at the slightest dilution, seldom surviving in the osmolarity of honey diluted to near 10% (Molan, 2012b). Still, the most common wound-infecting specie, *Staphylococcus aureus*, is exceptionally tolerant of osmolarity and can survive at honey concentrations of up to 30% (Molan, 2012b). With higher dilutions the antibacterial activity of honey is no longer attributed to its sugar content and is instead promoted by other compounds (Kwakman & Zaat, 2012).

1.4.2. pH of honey

Honey has a characteristic acidic pH range of 3.2 – 4.5, which in itself is capable of being inhibitory to several bacterial pathogens, with the acidity level changing according to botanical source and geographical nature (Mandal & Mandal, 2011; Satarupta & Subha, 2014; Vallianou, Gounari, Skourtis, Panagos & Kazazis, 2014). The minimum pH values for growth of common pathogenic bacteria were obtained in a study by Mandal & Mandal (2011), which evaluated *Escherichia coli* (pH 4.3), *Salmonella* spp. (pH 4.0), *Pseudomonas aeruginosa* (pH 4.4) and *Streptococcus pyogenes* (pH 4.5).

In addition and taking into account that *Staphylococcus aureus* is one of the most well known pathogens in terms of global health concerns, a study analyzing the interactions between lactic acid bacteria (LAB) and *S. aureus* inhibition also highlighted the role of pH, among other factors (Charlier, Cretenet, Even & Le Loir, 2009). Though this study essentially encompassed food systems and the vaginal environment due to the presence of LAB and consequent fermentation and acidification, the concepts can be applied in analogous form to honey. Charlier et al. (2009) deemed a pH of 4-4.5 as likely to inhibit *S. aureus*, seeing as its minimum growth pH is 4.6, with optimum growth being close to neutrality. Thus, with honey´s intrinsic even more acidic pH, it is logical to assume that it will also exert the same inhibitory action upon *S. aureus*. In addition, seeing as information regarding *Staphylococcus pseudintermedius* was not found, it is probable that the same concepts also apply, due to their similarity in nature.

However, the concentration of the acid itself in common honey is low and neutralization of this acidity takes place when honey is mixed with fluid from wounds or saliva. The surrounding environment of cells contains concentrations of bicarbonate, such that the dilution of common honey by an equal volume of extracellular fluid would elevate the pH to near neutrality (pH – 6.8) (Molan, 2012b). This would essentially nullify the acidity as a

contributor to antibacterial activity in situations where significant dilution takes place (Molan, 2012b). In this sense the greater part of honey´s antibacterial activity is not owed to its pH and is instead attributed to other properties.

1.4.3. Hydrogen Peroxide

Hydrogen peroxide (H_2O_2) is considered to be the major antimicrobial factor in the majority of honeys (Brudzynski, 2011; Mohaptra, Thakur & Brar, 2011; Molan, 2012b; White et al., 1963). There is correlation between levels of endogenous hydrogen peroxide and the extent to which inhibition of bacterial growth occurs (Brudzynski, 2006; White et al., 1963). The previously mentioned study by Charlier et al. (2009) reports on such inhibition, in this case specifically of *S. aureus*, by means of H_2O_2. The study refers to some lactobacilli strains being able to inhibit *S. aureus* growth, with bacteriostatic and bactericidal effects oscillating in accordance to different concentrations (Charlier et al., 2009).

The enzyme glucose oxidase, which is secreted by the honeybees directly into the nectar during honey production becomes activated with the moderate dilution of honey, upon which it converts the breakdown of glucose into gluconic acid and hydrogen peroxide, under aerobic conditions (Kwakman & Zaat, 2012). This previous notion was demonstrated through studies conducted by White et al. (1963), which observed that activity of hydrogen peroxide took place only upon exposure to air and dilution:

Glucose Oxidase + Oxygen → Gluconic Acid + Hydrogen Peroxide

This enzymic oxidation of the glucose occurs at a very slow rate in undiluted honey and increases significantly as the honey becomes diluted. The inhibine number of any given honey, as previously mentioned, is directly related to the hydrogen peroxide concentration produced in assay plates during inhibine assay procedures, by the honey enzymes (White et al., 1963).

The presumed function of H_2O_2 is to prevent the spoilage of honey when it is in unripe state, during which the sugar concentration is not yet at levels able to prevent microbial growth. During the ripening process glucose oxidase is inactivated and regains its activity upon dilution of honey (Kwakman & Zaat, 2012). The antibacterial activity attributed to H_2O_2, therefore, is determined by a balance between its activation through glucose oxidase and its neutralization, or absence, by the addition of catalase. The peroxide activity may also be destroyed by the presence of heat (Mandal & Mandal, 2011).

H_2O_2 concentration is determined by the relative levels of glucose oxidase, which are synthesized by the bee, as well as the enzyme catalase, originating from flower pollen. The latter is an enzyme that neutralizes H_2O_2, thus destroying its activity (Mandal & Mandal, 2011). Another study assessing the mechanisms by which honey kills bacteria through the successive neutralization of individual honey bactericidal factors (Kwakman et al., 2010) also demonstrated that the addition of catalase indeed reduced hydrogen peroxide to negligible values.

There is a delicate relation between the two enzymes, as mentioned previously, which might propel the idea that since glucose oxidase is produced by the bees, which maintain the process of ripening honey in strict and narrow limits, that honeys throughout the world may not differ so much in hydrogen peroxide. However, it is imperative to remember that catalase originates from plant sources, which translate to the amount of pollen retrieved by the bees and thus will ultimately determine the final levels of hydrogen peroxide (Weston, 2000). Furthermore, seeing as glucose oxidase is sensitive to external conditions, the antibacterial activity of honey that is hydrogen peroxide-dependent will depend on its exposure to heat and light during processing and storage (Molan, 2012b).

An example of the practical application of this peroxide activity is its use in wounds, where H_2O_2 contacts with the moist environment and the enzyme glucose oxidase is activated (Creemers & Bosma, 2006). Hydrogen peroxide alone for use in wound dressings has largely gone out of use due to its inflammatory effects and the risk of cytotoxicity (Molan, 2012b). Honey has demonstrated safe and effective antimicrobial activity through the continuous supply of low levels of hydrogen peroxide over an extended period of time, in contrast to a large amount at a single time (Bang, Buntting & Molan, 2003). A study conducted on 50% solutions of honey by Bang et al. (2003) showed that hydrogen peroxide accumulated to a peak level, after which it dropped, eventually to zero after 24-48 hours. This not only supported the fact that hydrogen peroxide would not accumulate to levels considered harmful to tissues but also alerted towards the notion that the antibacterial activity attributed to this substance was limited and that such concept would have to be applied in clinical use, for example in wound dressings, which would have to be changed with appropriate frequency (Bang et al., 2003).

1.4.4. Effect on Biofilms

A study by Merckoll, Jonassen, Jeansson & Melby (2009) addressed the effects of honey on ´planktonic´ bacteria on agar plates, or bacteria tested in its most vulnerable form, versus bacteria living in biofilm, a layer of bacteria-secreted polyssacharide commonly associated

with chronic infections. The latter are protected from the patient´s immune system and the action of antibiotics, with the ability to be 1000 times more resistant to antibiotics in contrast with the more vulnerable ´planktonic´ bacteria, which are used in laboratories for antibiotic sensitivity testing (Merckoll et al., 2009). The study, therefore, evaluated honey´s effects on typical real-life situations, such as recalcitrant wounds and the bacteria embedded in biofilm. In this case (Merckoll et al., 2009) the commercially available Medihoney® (Medihoney Pty Ltd., Queensland, Australia) was used, which is a mixture of gamma-irradiated honey that includes *Leptospermum* species, or Manuka honey. It was compared with the commercially available culinary local unmixed forest honey (Solhøy Bigård, Østfold, Norway). The bacterial strains utilized were two Gram-positive and two Gram-negative and included a methicillin-resistant *Staphylococcus aureus* (MRSA) strain isolated from a pus sample from a pediatric surgery department. It was found that both honeys slowed the growth of exponential phase bacteria from a concentration of as low as 0,8% (Merckoll et al., 2009). In terms of planktonic bacteria honey inhibited its growth even at very low concentrations and although the biofilm appeared to offer protection for the bacteria, the substances in honey were able to diffuse through the matrix, though higher concentrations were necessary. Though the Norwegian forest honey was not as effective as Medihoney® it still proved to be bactericidal. Nevertheless, culinary honeys should not be used in treating wounds since they are not sterile, as will be explained in further detail ahead.

Another study by Ansari et al. (2013) assessed honey´s *in vitro* effect on fungal biofilms, utilizing the common pathogen *Candida albicans*. The honey used for this experiment was jujube honey due to its known ability to decrease and disrupt mature biofilms. Results were obtained through scanning electron microscopy and atomic force microscopy, which indicated cellular morphological alterations on the structure of *C. albicans*, as well as a decrease in its biofilm thickness (Ansari et al., 2013). Such findings serve as illustration of the broad antimicrobial spectrum associated with honey in that it can also be considered an effective anti-fungal agent.

1.4.5. Effect on Colonization

As elucidated by Wolcott et al. (2010), though bacterial colonization of a wound is not necessarily considered as being detrimental to the wound healing process *per se*, it may lead to chronic infection if the bacteria persistently utilize the host´s defenses to the point of exhausting their immune system´s protective capacities (as cited in Westgate & Cutting, 2013, p. 1). Furthermore, Jørgensen et al. (2006) indicates chronic wound infections as being

responsible for considerable patient morbidity in association with decreased quality of life (as cited in Westgate & Cutting, 2013, p. 1).

Though *in vitro* studies regarding the antibacterial effects of honey exist in large numbers there are few in which honey´s activity is assessed on healthy subjects and tissues. Kwakman et al. (2008) did just this and not only studied Revamil® (Bfactory, Netherlands), a medical grade honey and its bactericidal spectrum but also observed its efficacy in reducing microbial skin colonization in healthy human volunteers. A number of microorganisms were subjected to the honey, including *Escherichia coli*, *Pseudomonas aeruginosa*, clinical isolates of *Enterobacter cloacae* and *Klebsiella oxytoca*, extended-spectrum beta-lactamase (ESBL) – producing strains of these, as well as methicillin-susceptible and methicillin-resistant strains of *Staphylococcus epidermidis* and *S. aureus*, among others. Results showed that the honey had reproducible bactericidal activity against both antibiotic-resistant and susceptible isolates (Kwakman et al., 2008).

Regarding the healthy human volunteers, this study (Kwakman et al., 2008) included an investigation to assess decrease in skin colonization on the forearm. For such, 2 patches of skin were used, over which 0,5 ml of honey was applied to one area and covered with a transparent polyurethane dressing, with the other remaining as the control. After 2 days the honey was collected from the patches and it was found that honey-treated patches showed significantly less colonization than did the corresponding untreated control patches, which instead demonstrated increased colonization (Kwakman et al., 2008).

1.4.6. Effect on angiogenesis

A study by Rossiter, Cooper, Voegeli & Lwaleed (2010) investigated the possibility of honey as an angiogeneic agent through use of *in vitro* analogues of angiogenesis and an endothelial proliferation assay, as well as possible cytotoxicity. In this case the types of honeys evaluated were as follows: an artificial honey solution of glucose and fructose, a common supermarket honey, Activon® (Advancis Medical Ltd, UK), which is medical grade Manuka honey and Mesitran® ointment (Triticum, Netherlands), which is also a medical grade honey product based on hypoallergenic lanolin, among other components (Rossiter et al, 2010). The results of the cytotoxicity assay revealed that only Activon® showed a significant dose-response (Rossiter et al., 2010).

With regard to the angiogenesis assay, which was based on a rat aorta ring, tubule formation was evaluated with basis on density and total length. Results for all products were similar and showed highest pro-angiogenic effect at 0,2% v/v honey. Regarding endothelial proliferation, based on multi-well plates containing endothelial cells, activity was evaluated through

photographs and analysis of the wells after staining in order to measure pseudotubule density and branching. Honey dilutions at 0,2% v/v and 0,04% v/v were found to be pro-angiogenic, while at 1% they were neutral and at 5% they were anti-angiogenic. The potential which honey has towards angiogenesis is "severely under-investigated" (Rossiter et al., 2010, p. 16) and thus this laboratory model provided a foundation for future studies.

2. ALL HONEYS ARE NOT THE SAME

2.1. Honey use in medicine

It is only in recent times that the medical profession has turned its attention once more to the ancient practice of using honey as a medicinal agent. However, many present-day practitioners are unaware that honeys vary greatly with regard to their therapeutic potential and that some are more suitable than others. Oftentimes honey is treated as a "generic medicine" among scientists and physicians, with all of the variations being overlooked (Molan, 2012c). The protocol used for honey in wound management, for example, is highly variable and depends on the clinician´s preferences, with some purchasing inexpensive honeys intended for consumption, while others opt for standardized irradiated medical honey (Carnwath, Graham, Reynolds & Pollock, 2013). Although it is fact that natural honey originating from the comb has antibacterial properties, it is not of medical grade and is thus contra-indicated for wound care. "All honeys are not the same and do not possess the same therapeutic advantages; therefore, honey should not be considered as a generic term" (George & Cutting, 2007). Further yet, the antibacterial potency among numerous honeys varies in accordance with the inhibine number, or number of dilution steps a sample of honey could be subjected to while still retaining antibacterial activity. Such variance among honeys calls for careful study prior to their selection for use in clinical treatments. Nevertheless, much of the published research, whether clinical or microbiological, has been conducted without knowledge of the actual antimicrobial activity, with the minimum inhibitory concentration (MIC) sometimes varying 100-fold between different honeys (Molan, 2012b).

A study by Cooper & Jenkins (2009) compared the antibacterial activity of 17 samples of table honey purchased from British supermarkets with medical grade Manuka honey (Manukacare® 18+, Comvita, UK). The inhibitory potential of each honey sample was estimated through determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against 6 bacterial cultures, as follows: 2 laboratory reference cultures – *S. aureus* NCTC 6571 and *E. coli* NCTC 10418, as well as 4 clinically isolated bacteria from chronic wounds – MRSA, *Streptococcus pyogenes*, *S.*

epidermidis and *P. aeruginosa*. The results of this study (Cooper & Jenkins, 2009) revealed antibacterial activity in 9 of the honey samples, with the medical grade honey having the greatest bactericidal action against the tested cultures.

In addition, a wide variety of microorganisms were recovered from the 18 table honeys (Cooper & Jenkins, 2009). Though most were mesophilic aerobic bacteria and were not usually considered to be pathogens, there were some capable of colonizing chronic wounds, such as *Clostridium ramosum* and *Staphylococcus warneri*. Bacillus species were the most recovered and were present in 14 samples of honey. No organisms were detected in the medical grade honey, as the sample complied with specific regulations, having been irradiated for sterilization. In summation, this study interestingly showed that not only is the antibacterial activity of common culinary honeys unreliable and sometimes non-existent, but the potential presence of pathogenic organisms in them limits their use in medicine (Cooper & Jenkins, 2009).

With regard to the specific utilization of honey in the treatment of wounds, though it may be generally assumed that even honeys with low antibacterial activity will be suitable, it is imperative to consider the factors which will alter the microenvironment and hence the potency of the honey. In open wounds fluid will seep out and dilute the honey, thus decreasing its activity and when infections are in the mouth or stomach, saliva and gastric fluid will dilute the honey and also decrease its action (Molan, 2012c).

2.2. Manuka honey

The currently available research on honey´s action pertains mainly to two principal groups which vary in respect to the component involved in antibacterial activity. These are the European and American honeys, possessing catalase-sensitive activity and correlation with internal hydrogen peroxide and the *Leptospermum* spp honeys, which are independent of hydrogen peroxide and are instead active with basis on an internal component named methylglyoxal (Brudzynski, Abubaker & Wang, 2012). Manuka honey is set apart from other honeys in that it is a non-peroxide honey, therefore retaining its full antibacterial activity in the presence of catalase and in contrast to other honeys (Molan, 2012g). It is produced in most abundance in New Zealand, where Manuka trees grow uncultivated over large territory but is also produced in Australia, although in much lesser quantities, from *Leptospermum scoparium* trees (Molan, 2012g).

Though Maori tribes used manuka honey as a medicinal agent, its principal antibacterial component, methylglyoxal, is not integrated in the nectar collected by the bees to make honey and is instead formed by a chemical reaction occurring after the bees have processed the

nectar into the honey (Molan, 2012g). Manuka honey is currently being sold with four different rating scales of antibacterial activity and even when the same scale is used, variations can result due to different laboratory procedures, for example. (Molan, 2012g).

2.3. Medical grade honey

The term ´medical grade honey´ is largely used as a marketing term and there is no official definition as to what it is or what it must be. True medical grade honey must comply with a number of strict criteria and differs greatly from common table honeys, as previously mentioned. As elaborated by Postmes, van den Bogaard & Hazen,

> Like other natural products, the composition of honey is not constant, and, moreover, it may contain residues of pesticides or drugs such as tetracyclines that are used for treatment of bee diseases. In most countries honey for human consumption must be checked for residues; however, for medical use higher quality standards are needed. It seems advisable to use only honey derived from specific-pathogen-free (SPF) hives, which have not been treated with drugs, and are gathered in areas where no pesticides are used. Honey intended for medical use should be sterile and free of residues, which might make the clinical use of honey more acceptable (The Lancet, 1993, p. 756).

Regardless of the existence or absence of an official definition for what medical grade honey is, it is imperative that it be subjected to measures which will guarantee its safe clinical use, one of which is the sterilization procedure through gamma irradiation (Postmes et al., 1993; Molan & Allen, 1996). Furthermore the various types of honeys produced under this category undergo, in addition to the sterilization, careful filtration and are produced under exacting standards of hygiene (George & Cutting, 2007). In recent years numerous medical grade honey products composed of different types of honeys at different concentrations have been approved by the European Union for use in wound care and are being employed successfully (Stobberingh & Vandersanden, 2010).

2.3.1. Variation among medical grade honeys

Despite proven efficacy of the numerous commercially available medical grade honey products and their benefits over common table honeys, there are differences among the former regarding antibacterial activity. Stobberingh & Vandersanden (2010) set out to compare the *in vitro* activity of commercially available products against clinical isolates containing antibiotic resistant strains of *S. aureus* and *P. aeruginosa*. Among the products evaluated were pure honey formula Revamil® (B-factory, NL), pure Manuka honey (Activon, UK), L-Mesitran® Soft, containing 40% honey (Triticum, NL) and L-Mesitran® Ointment, containing 48%

honey (Triticum, NL). Results for the study (Stobberingh & Vandersanden, 2010) revealed that Revamil®, for example, was bactericidal against *P. aeruginosa* but was not effective against *S. aureus*. Moreover it was demonstrated that L-Mesitran® Soft was by far the most effective of the products in terms of antibacterial activity and required the least amount of product to obtain such efficacy, meaning that potential was observed at the lowest concentration in comparison to others. The other products would need relatively more material in order to reach the same level of antibacterial activity, with the exception of Revamil®, which showed no significant activity against *S. aureus* (Stobberingh & Vandersanden, 2010).

2.3.2. Risks & gamma irradiation

The risk most notoriously associated with honey use is that of botulism due to the presence of clostridial spores and gamma irradiation has been found to kill any such spores, allowing for a sterile product without loss of antibacterial activity (Merckoll et al., 2009). Furthermore, Creemers & Bosma (2006) advise against the use of common retail honey in treating wounds, as it is probable that these may contain traces of pesticides and herbicides which may pose a risk of toxicity. In addition to pesticides and herbicides, Stobberingh & Vandersanden (2010) also mention other dangers such as heavy metals and antibiotics used to treat diseases in bees.
Seeing as it has been well established that honey´s antibacterial activity is heat labile, sterilization through autoclave would not be viable and would thus destroy its beneficial characteristics. Postmes et al. (1993) utilized gamma irradiation on 2 batches of lime honey, thus contributing to the viability of honey use in a clinical context. One batch contained 520 colony-forming units (CFU) *per* 100 grams of honey, with 40 CFU identified as *Clostridium perfringens* and the rest *Bacillus* spp, with the other batch containing 4200 CFU *Bacillus* spores *per* 100 grams. Irradiation with 18 kGY successfully sterilized both batches without compromising their antibacterial activity (Postmes et al., 1993). Molan & Allen (1996) also investigated the effect of gamma irradiation (25 kGy) on the same antibacterial activity of honey and found that there were no significant changes in such. In the aforementioned study (Molan & Allen, 1996) 5 honeys were utilized, with 2 having their activity attributed to hydrogen peroxide and the other 3 being manuka honeys with non-peroxide activity. The honeys were tested against *S. aureus* in an agar well diffusion assay and even when doubling the radiation to 50 kGy antibacterial activity was maintained. Nevertheless, testing of honey containing spores of *Clostridium perfringens* and *Clostridium tetani* indicated that sterility was achieved at 25 kGy (Molan & Allen, 1996.)

In essence, true medical grade honey must be able to guarantee its clinical safety through the elimination of potential pathogenic organisms by gamma irradiation, without compromise of and whilst preserving its full antimicrobial and healing properties.

2.4. Honey as a medical device

The use of honey, as previously mentioned, has been recorded throughout history and it appears to have been a standard form of treating wounds until the appearance of antibiotics in the 1940s. It has even been said by doctors in some reports, that the idea of re-using honey was provided by older nursing staff, who recalled it being used in the past (Molan, 2012d). As more research arises regarding the antimicrobial properties of honey, an alternative medicine branch named apitherapy has been developed, which offers treatments based on honey and other bee derivatives against many conditions, including bacterial infections (Mandal & Mandal, 2011).

Following the increasing publications and reports in medical journals regarding honey´s favorable results in the clinical environment, particularly in wound dressings, two developments resulted according to Molan (2012d): practitioners adhered to the use of honey in wounds and companies started to produce sterilized honey wound dressings as registered medical devices. The European Commission´s Directorate General for Enterprise states that,

> Medical devices are defined as articles which are intended to be used for a medical purpose. The medical purpose is assigned to a product by the manufacturer. The manufacturer determines through the label, the instruction for use and the promotional material related to a given device its specific purpose. As the directive aims essentially at the protection of patients and users, the medical purpose relates in general to finished products regardless of whether they are intended to be used alone or in combination (Medical Devices: Guidance document, 1994, p.3).

Within the vast field of products that can be considered medical devices, medical grade honey products are under the category for non-invasive devices, which are further classified in accordance to specific European Commission´s Directorate General for Health and Consumer. In general the medical honey products follow rule number 4 of the Commission, which addresses devices “In contact with injured skin (mechanical barrier, compression, absorb exudates)” (European Commission´s DG Health and Consumer, 2010, p.17), which then leads to a final classification of IIb, that according to the Commission is “Intended for wounds which breach dermis and heal only by secondary intent” (European Commission´s DG Health and Consumer, 2010, p.17).

2.5. Generalities in wound healing

Molan (2012e) simplifies the wound healing process so as to understand how honey works in promoting it. The rate-limiting factor in healing is oxygen supply from newly formed blood capillaries, since oxygen does not dissolve in at a fast enough speed from the small surface area of the wound. New cells can only grow in a moist environment, such that if a wound dries out the surface is covered by a scab formed from dried wound fluid and new tissue can only grow beneath this. The result is a delayed overall repair process and a scarred surface where the scab used to be since no repair took place in the dry area (Molan, 2012e). Honey promotes granulation, epithelialization, as well as reduces the amounts of exudate (Al-Waili, Salom & Al-Ghamdi, 2011) and keeps bacteria out of the wound, as will be discussed.

Supporting results are also found in another study using honey for the topical treatment of skin wounds in mice (Ghaderi & Afshar, 2004). Formation of granulation tissue and activation of fibroblasts was increased by honey, in addition to greater thickness of the basement membrane and epidermis, as well as of the collagen fibers. In comparison with the control group, which received a simple dressing with sterile gauze, the honey-treated group demonstrated constant advantage in terms of absence of inflammation, edema and dehiscence. Final conclusions of this study were that honey can accelerate the wound healing process whilst increasing resilience, tensile strength and toughness of wounds (Ghaderi & Afshar, 2004).

2.5.1. Physical barrier

Honey´s viscosity alone serves as a physical barrier which prevents bacteria from entering and keeps the wound moist in order to potentiate healing. In addition the high sugar content promotes the formation of a liquid layer between the wound surface and the dressing, since fluid is drawn out by means of osmosis. The resulting environment not only accelerates the healing process but prevents scab formation, thus avoiding scarred surface tissue. In contrast, dry dressings adhere to the surface of wounds and are a constant hindrance to tissue renovation when changed, as the newly formed tissue is torn off the surface (Molan, 2012e).

2.5.2. Wound acidification

Molan (2012e) also mentions the beneficial acidifying action of honey on wounds, which has been found to accelerate the healing rate. There are two mechanisms stated in his work, one of which regards the release of more of the oxygen that is being carried by hemoglobin in the bloodstream, seeing as oxygen is the rate-limiting factor in new cell growth, as previously mentioned. The other involves the inactivation of the digestive enzymes in the wound, which may be responsible for the destruction of newly repaired tissues or the growth factors required

in order to stimulate new tissue formation. Such enzymes operate at a neutral pH and therefore honey´s more acidic pH inactivates their activity (Molan, 2012e).

2.5.3. Debriding action

"Debriding is the medical term for removing from the surface of a wound any attached pus and/or dead tissue" (Molan, 2012e). This process is essential to wound healing, as the remaining of pus or dead tissue on a wound will generate an ongoing inflammatory response and thus prevent permanent healing. In addition to the inflammatory response the presence of pus provides a favorable environment for bacteria to proliferate. Honey is a rapid and effective debriding agent in comparison to other pharmaceutical products claiming the same action (Molan, 2012e; Morris, 2008). The explanation for such is that honey stops white blood cells from producing plasminogen activator inhibitor (PAI) that normally prevents the activation of plasmin in wound tissue. These clots are responsible for the attachment of pus and debris to the wound surface. Since the honey stops the production of PAI, it allows for more plasmin to be activated and thus digest the fibrin clots, culminating in the removal of pus and debris (Molan, 2012e).

2.5.4. Deodorizing effect

The foul odor commonly associated with infected wounds, particularly when anaerobic bacteria are present, has been motive for discomfort among patients and honey exerts rapid effect in eliminating it (Al-Waili et al.; 2011; Molan, 2012e; Morris, 2008). The unpleasant odor is associated with the breakdown of proteins in wound tissues by bacteria, which generates sulphur compounds and amines. Honey swiftly deodorizes wounds by simply providing the bacteria with glucose as a source of alternative energy, which is preferred over protein and through which no malodorous components arise (Molan, 2012e).

2.5.5. Anti-inflammatory effect

The exact mechanisms through which honey exerts anti-inflammatory action have yet to be studied and clarified but the evidence for such is large and continuously expanding in the scientific world. A study comparing the effectiveness of Indonesian honey, Manuka honey and a control hydrocolloid dressing on the rate of wound healing in mice (Haryanto, Urai, Mukai, Suriadi, Sugama & Nakatani, 2012) revealed both honeys to have the upper hand. The mice had induced wounds which were treated with the 3 comparative substances and evaluated throughout 14 days. Macroscopic results on days 2, 5 and 7 showed smaller wound areas in both honey groups, with newly formed granulation tissue and epithelium, whereas the control group had larger wound areas. Microscopic analyses on day 3 revealed greater

numbers of neutrophils in the honey groups, with corresponding redness in the surrounding areas, all which disappeared on day 7. The control group, on the other hand, presented periwound edema and increased neutrophils on day 7. Myofibroblasts and new capillaries were detected on day 3 in both honey groups, a much faster rate than that in the control group. The study (Haryanto et al., 2012) showed that inflammatory processes had a shorter duration and were more rapidly depressed in honey-treated groups than in the hydrocolloid dressing group.

Another interesting study compared the anti-inflammatory and antioxidant effects of a gamma-irradiated honey in treating alkali injury in the eyes of rabbits, with that of conventional treatments (Bashkaran, Zunaina, Bakiah, Sulaiman, Sirajudeen & Naik, 2011). Results showed that honey treatment was as effective as the conventional one, which in this case consisted of topical prednisolone acetate 1%, ciprofloxacin 0,3% and oral ascorbic acid (Bashkaran et al., 2011). On a macroscopic level the honey had equal effect on the conjunctival hyperemia, corneal edema and epithelial healing resulting from the induced alkali injury. On a histopathological level, rabbits from both of the compared groups showed only mild corneal infiltration by polymorphonuclear cells, showing that the honey indeed does have an anti-inflammatory potency comparable to that of other medical treatments (Bashkaran et al., 2011).

It has also been suggested that honey´s anti-inflammatory effect is related to the stimulation of cytokines from monocytic cell lines, which are known to integrate healing and tissue repair (Tonks, Cooper, Price, Molan, & Jones, 2001; Tonks, Cooper, Jones, Blair, Parton & Tonks, 2003). The effects of honey on the release of important cytokines from a model of honey-treated MonoMac-6 (MM6) cells were investigated and results revealed significant increases in pro-inflammatory mediators tumor necrosis factor-α (TNF-α) and interleukin (IL)-1β, as well as anti-inflammatory mediator IL-6 when compared with untreated or artificial honey-treated cells (Tonks et al., 2003).

In addition, recent research in New Zealand has revealed that anti-inflammatory action can also be attributed to a protein named Apalbumin 1, which bees incorporate into the nectar they collect during the process of making honey (Molan, 2012h). Apalbumin 1 suppresses the initial phase of inflammation, which involves white blood cell action over bacteria and other particles. Furthermore it has also been found that methylglyoxal, the major antibacterial component of manuka honey, raises the efficacy of Apalbumin 1 in white blood cell suppression (Molan, 2012h).

2.5.6. Antitumor effect

An *in vitro* study evaluating the effects of Jungle honey on immune function and induced tumors in mice revealed promising results (Fukuda, Kobayashi, Hirono, Miyagawa, Ishida, Ejiogu, Sawai, Pinkerton & Takeuchi, 2011). Jungle honey is obtained by the wild honeybees of the Nigerian tropical rainforest and is used in traditional and preventive medicine (Fukuda et al., 2011). Mice were firstly injected daily with jungle honey, for 7 days via the intra-peritoneal route, after which peritoneal cells were obtained. Mice in the control group were injected with phosphate buffered saline (PBS) and peritoneal cells from such were also obtained. The number of cells obtained was increased 4-fold in the group injected with honey versus the control group (Fukuda et al., 2011). New cell populations in the honey group were also present, which upon isolation, were found to be neutrophils. Next, for the investigation of antitumor activity by immune cells, Lewis Lung Carcinoma/2 (LL/2) tumor cells were used in a syngeneic manner (Fukuda et al., 2011). The incidence of LL/2 tumors was only 20% in the honey-injected mice and 100% in the control group, with the mean tumor weight also heavily decreased in the former group. These results suggested the preventive effect of honey on tumor growth. Furthermore, reactive oxygen species (ROS), an antitumor factor, was also evaluated and found to be significantly increased in the honey group. It is possible that the antitumor activity of the honey can be attributed to the production of ROS by infiltrated neutrophils into the tumor tissue (Fukuda et al., 2011).

2.5.7. Immuno-stimulatory effect

According to Molan (2012e), studies have been conducted in wounds in which no bacterial infection nor persistent inflammation was present, in order to evaluate the effect of honey on healing. Such studies revealed that honey nearly doubles the rate of healing in comparison with cases in which it was not applied and that it exerts effects on cellular elements of immunity and antibody production (Al-Waili et al., 2011; Molan, 2012e).

In the previously addressed study in mice by Fukuda et al. (2011), honey demonstrated chemotactic activity for the neutrophils. In addition, cell numbers, migration and velocity were significantly higher than in the control group.

2.5.8. Antioxidant activity

"Free radicals are very reactive chemicals which react with and change other molecules next to them" (Molan, 2012f, p. 1). They are in constant formation, arising from diverse sources such as chemicals ingested in food or present in polluted air, as well as during exposure to gamma rays and X-rays. The free radicals formed within the body are mostly a result of metabolism, mainly from the breakdown of hydrogen peroxide. Free iron atoms are

responsible for the formation of radicals from hydrogen peroxide in that during certain situations, for example infection, injury, inflammation or alcoholism they are released from the proteins which bind them and are catalyzed. These unbound iron atoms forming free radicals are responsible for most consequences of aging (Molan, 2012f).

Antioxidants are substances which work against free radicals and act by stopping a chain reaction or initial formation, with the latter being more effective (Molan, 2012f). They prevent disease through the scavenging of reactive oxygen species, reduction of peroxides and repair of oxidized biological membranes (Bashkaran et al., 2011). Honey contains aqueous and lipophilic antioxidants, therefore allowing it to act on various cellular levels, exerting a more ample antioxidant effect. This type of activity is related to certain characteristics of honey and it is established that higher water content and darker color equate to greater presence of antioxidants (Bashkaran et al., 2011).

2.5.9. Honey *versus* Silver

Silver is also considered to be a topical antibacterial agent and silver-impregnated dressings are used extensively in the clinical setting when treating chronic wounds (Du Toit & Page, 2009; Westgate & Cutting, 2013). Like honey it can be delivered to the wound environment in different forms, which relate to its antimicrobial efficacy (Westgate & Cutting, 2013). According to Beam (2009), silver ions work by binding themselves to bacterial cell walls and enzymes, triggering reactions which disrupt internal mechanisms, disrupting cell wall and preventing cell replication, leading to death (as cited in Westgate & Cutting, 2013, p. 2). However, unlike honey use, silver is associated with cell toxicity when used in high levels or for prolonged time frames, although discontinuation of treatment does rapidly reduce symptoms (Schaller, 2004, as cited in Westgate & Cutting, 2013, p.2).

Du Toit & Page (2009) conducted an *in vitro* comparison of cell toxicity between honey and silver dressings on human skin keratinocytes and dermal fibroblasts. For this study (Du Toit & Page, 2009) the product chosen to represent honey used by practitioners was L-mesitran® Hydro (Triticum, Netherlands), an organic, monofloral and hydroactive medical-grade honey dressing. The honey product yielded high cytocompatibility with tissue cultures in comparison with the silver dressing, which was associated with high cell toxicity. Honey proved to stimulate early proliferation of both keratinocytes and fibroblasts and even after one month of continuous stimulation there was no noticeable over-proliferation of the honey-treated cells when compared with the other cultures (Du Toit & Page, 2009).

The cells exposed to the silver product showed poor proliferation, with cell survival, migration and shape having been negatively affected. In addition, cell numbers declined

throughout the course of time, resulting in no surviving keratinocyte or fibroblast cultures or monolayers at three weeks, thus indicating presence of continuous cell toxicity (Du Toit & Page, 2009). Yet despite the favorable results yielded by the honey product and the clear advantage over silver dressings, it is crucial to keep in mind that "The mechanism by which honey enhances cell stimulation *in vitro* is still open to speculation" (Du Toit & Page, 2009, p.8) and more studies concerning this aspect should be conducted in order to further solidify honey implementation in wound treatments.

3. L-MESITRAN®

3.1. Triticum®

The Dutch Triticum company was founded in the year 2000, based on the vast research conducted throughout many previous years by Dr. Theo Postmes, Phd in Biochemistry, regarding the properties of honey. Through this extensive research and numerous publications the company launched unique and patented products in 2002, bearing the European Conformity (CE) marking, making the L-Mesitran® gamma the first honey-based wound care product line in Europe. In 2007 the company´s dressing products were cleared by the Federal Drug Administration (FDA) (Triticum®, 2012).

3.1.1. Characteristics

In vitro tests reveal that the most common wound bacteria, including *S. aureus*, *P. aeruginosa* and even the common fungal pathogen *C. albicans* will be killed within 24-48 hours of L-Mesitran® usage. *In vivo* results are corresponding and after treatment, wounds will be cleared of most bacteria, including MRSA and vancomycin-resistant enterococci (VRE) bacteria (Triticum®, 2012).

3.1.2. Applications – Human medicine

Honey is a particularly attractive alternative to antibiotics and even silver in wound management, as it is safe and lacking in adverse effects, while simultaneously providing cost effective therapy through lowering cost of materials (Stobberingh & Vandersanden, 2010). L-Mesitran® wound dressings were well tolerated, safe and effective in a post-operative wound dehiscence following total laryngopharyngectomy in a patient with an advanced hypopharyngeal tumor (Pereira, Ângelo & Ferreira, 2012). The authors of the previous study continue to utilize honey-based wound dressings as alternative and experimental therapy at the Portuguese Oncology Institute, with the simultaneous objective of implementing them as standard treatment protocol for the aforementioned type of post-operative wound (Pereira et

al., 2012). Another study by Pereira et al. (2013) successfully used honey ointment (L-Mesitran®) to manage supra-tracheostomy necrosis after laryngectomy in an oncologic patient at the Portuguese Oncology Institute.

It seems pertinent to provide visual results regarding the use of medical honey, seeing as some are truly remarkable. As the use of honey is regaining its momentum in the medical world, oftentimes reports are not enough and images hold greater impact. An example of success in the face of antibiotic resistance in human medicine was the case of a patient who presented with a post-surgical infection on their left leg, which tested positive for *Staphylococcus aureus* (Fig.1). This patient received systemic antibiotic treatment for 2 weeks but the wound worsened and presented with exudate and necrotic tissue (Fig.2). The antibiotics were thus stopped and the patient started treating herself with L-Mesitran® Ointment and Border dressing. Within 1 day of commencing treatment (Fig.3 and Fig.4), the necrotic tissue had subsided due to the autolytic debridement promoted by the honey. By approximately 3 weeks of treatment at home, the wound was completely healed (Fig.5) (Aaftink, 2008).

Fig. 1 - Post-surgical infection. **Fig. 2** - Post-antibiotic treatment. **Fig. 3** - Start of L-Mesitran®.

Fig. 4 - 1 day after L-Mesitran®.

Fig. 5 - Healed at 3 weeks.

(All photographs kindly provided by L-Mesitran®).

Another case report involved a skin tear (Fig. 6) in a 90 year-old patient in otherwise good health (Kegels, 2008). The wound was treated daily by a nurse with L-Mesitran® Soft, due to its more gentle properties and then covered with a non-adhering dressing. The honey kept the environment moist and quickly debrided and epithelialized the wound. In addition, the patient did not experience any discomfort or adverse effects (Kegels, 2008). Photos were taken at approximately 2-week intervals (Fig. 7 & Fig. 8), with the wound having healed after nearly 1 month from presentation.

Fig. 6 - Start of L-Mesitran®. **Fig. 7** - 2 weeks after L-Mesitran®. **Fig. 8** - Healed at 1 month.



(All photographs kindly provided by L-Mesitran®).

Other successful applications include treatment of foot fungi involved in tinea pedis, such as *Trychophyton* sp. and *Microsporum* sp. (Van den Oord, 2008), progressive, painless healing of a complicated wound associated with Diabetes Mellitus (Den Besten, 2004), successful debridement of a MRSA-infected surgical wound (Owen, 2005), which prevented amputation and improvement of severe pediatric burns (Smaropoulos, 2007), among countless others.

3.1.3. Applications – Veterinary medicine

The implementation of honey in treating animals is as equally valid as it is with humans and the applications are just as vast. For example, surface pyoderma, a commonly observed skin disease in dogs, is usually treated topically with antibacterial shampoos and/or topical antibiotics. A randomized pilot study by Jakobsson (2011) evaluated 40 affected skin areas from 29 dogs with pyoderma, some of which were assigned 3% chlorhexidine shampoo (Pyoderm®) and others honey-based ointment (L-Mesitran®). Results showed that the latter was safe and as effective as the shampoo treatment, with pet owners having considered it easier to use when compared to washing with a shampoo (Jakobsson, 2011).

In keeping with the importance of visual results regarding honey´s medical potential, animals have also provided impressive images. A tortoise was found badly wounded, under the suspicion that it had been attacked by a dog (Widmann, 2011). There was heavy

contamination with soil and presence of maggots, with the left forelimb having been severed at the middle of the humerus. There was damage to the carapace, particularly in the rostral portion and plastron bone. The skin on the breast had been completely detached until the neck. The injured limb was amputated (Fig. 9) at the shoulder joint and for the next 3 weeks after bathing, L-Mesitran® Soft was applied to the site. Weekly debridement under light sedation was also done in order to remove non-vital shell parts. After 1 month of covering the area with honey-based gel there was excellent wound healing (Fig. 10). With 6 weeks of intensive home nursing the tortoise was released fully healed (Fig. 11) (Widmann, 2011).

Fig. 9 - Post-amputation & start of L-Mesitran®.

Fig. 10 - After 1 month of treatment.

Fig. 11 - Fully healed at 6 weeks.

(All photographs kindly provided by L-Mesitran®).

4. OTITIS

4.1. General prevalence

There is a wide range of published studies stating that within the veterinary field, dermatology consultations make up between 17% and 25% of all small animal consultations (Khoshnegah, Mavassaghi & Rad, 2013). Ear disease is also inserted in the dermatological context, with otitis specifically accounting for up to 15% of all dogs presented for veterinary care (Miller, Griffin & Campbell, 2013).

5. OTITIS EXTERNA

5.1. External ear anatomy

With regard to embryonic origin the external ear canal is formed by the groove which lies in between the first and second pharyngeal arches, with the arches expanding laterally so as to form the wall of the canal and the pinna. The tympanic membrane is formed by apposition of endoderm and ectoderm (Fletcher & Weber, 2013).

The external ear is comprised by the pinna and external acoustic meatus and serves to collect and locate the origin of sound waves. The pinna is a flared extension of the auricular cartilage and is covered by skin, which is more firmly adherent on the concave portion. The portion forming the body of the pinna is the scapha and the free edges are termed rostral border of the helix and caudal border, respectively. The antihelix is the medial ridge with the prominent tubercle situated on the medial aspect of the entrance to the vertical ear and opposite the antihelix is a dense plate of cartilage, the tragus. This extends caudally and medially to the antitragus and creates the caudal boundary of the opening into the external acoustic meatus (Harvey, Harari & Delauche, 2005).

The external auditory meatus is contained within the vertical and horizontal portions of the external ear canal. Proximally, it is adjacent to the tympanum and distally it is defined within the medial faces of various cartilaginous components at the base of the pinna. The size of the vertical canal correlates with body weight and the lumen becomes progressively narrower proximally (Harvey *et al*., 2005).

5.2. Pathogenesis

The external ear canal is lined by an extension of the surface integument toward the tympanum, thus being equally susceptible to the changes and diseases affecting skin anywhere else on the body (Rosser, 2004). Otitis externa consists of inflammation of the external ear canal, distal to the tympanic membrane, with possible involvement of the ear pinna (Moriello, 2013). It may be of acute or chronic nature, unilateral or bilateral, essentially with an underlying reason and multifactorial etiology (Bugden, 2013; Moriello, 2013; Rosser, 2004).

Primary causes, often aided by predisposing factors, are responsible for initiating the auricular inflammatory process, which is then maintained by common otic pathogens, such as *Malassezia* spp yeast and coagulase-positive *Staphylococcus* spp bacteria (Reeder, Griffin, Polissar, Neradilek & Armstrong, 2008). It is important to note that these bacteria and yeast organisms are invariably opportunist agents and cannot be considered the primary pathogens or solely responsible for any given case of otitis (Rosser, 2004).

5.3. Prevalence

Furthermore numerous skin pathologies, namely canine atopic dermatitis (CAD), present with associated otitis externa in 43% of cases (Favrot, 2009). Dogs with CAD, for example, account for a disproportionate percentage of animals that present with otitis externa, with the ear canals sometimes being the only region affected in some patients (Plant, 2009). In addition, food allergies are also closely correlated with recurrent otitis externa, as 56% to 80% of such allergic dogs present with this complaint (Jackson, 2009). Banfield Hospitals in the United States revealed that its clinical database indicated 18.2% of sick dogs attending their practices as being diagnosed with otitis externa and 8.9% of healthy dogs during their wellness exams demonstrated clinical signs of the disease (McQuillan, 2005). In addition, a very recent study by O´Neil, Church, McGreevy, Thomson & Brodbelt (2014) goes on to classify otitis externa as the most prevalent cause of visits to primary care veterinary practices. Yu (2013) adds that the prevalence of otitis externa among dogs ranges from 10-20% and perhaps even as high as 30-40% in tropical and subtropical environments around the world.

5.3.1. Predisposition

Predisposing factors are already present prior to the development of ear disease but end up increasing the risk for development of otitis externa, as well as impeding the normal protective mechanisms of the ear canal (Moriello, 2013; Restrepo, 2013; Rosser, 2004). When comparing dogs with cats, it is clear that the former are more predisposed to ear disease, as the auricular anatomy is very different among both, in that cats have upright pinnae, or erect ears, thus creating a less favorable environment for infection (Fontaine, 2009; McQuillan, 2005). Dogs with long and pendulous ears tend to present more ear disease than those with short, erect ears and include Golden Retrievers, Labrador Retrievers, Cocker Spaniels, Basset Hounds and Irish Setters. Other dog breeds are known to have specific physical features that predispose them to otitis, for example Shar-Peis whose ear canals are stenotic. Poodles, Lhasa Apsos and other heavy-coated breeds are associated with high density of compound hair follicles in their ear canals and Labrador Retrievers and Spaniels are both breeds with increased apocrine and ceruminous glands, thus producing greater quantities of earwax (McQuillan, 2005).

Other factors which can contribute towards generating an unfavorable microenvironment within the ear include iatrogenic trauma from possible excessive cleaning or over-treatment that can lead to maceration of ear tissue. Obstruction of the ear canal can also occur due to tumors or polyps, thus allowing for the pathological process to instill (Rosser, 2004).

Whether it be a narrowed, dense or infiltrated ear canal, all of these factors greatly contribute to create the perfect conditions for growth of organisms capable of generating ear disease (McQuillan, 2005). If and when possible, the predisposing factors should be addressed because although they are not able to cause ear disease alone, as they not only increase the risk for its development, as previously mentioned, they may largely hinder successful treatment (Bloom, 2009; Restrepo, 2013). Table 1 lists the general predisposing factors with basis on work from the aforementioned authors.

Table 1 – Common predisposing factors of otitis externa

þ Ear conformation/type	þ Anatomical stenosis
þ High density of compound hair follicles	þ Increased apocrine/ceruminous glands
þ Iatrogenic trauma	þ Over-treatment
þ Obstruction by polyp/tumor	

5.3.2. Primary causes

The primary cause is essentially the underlying cause and is important in determining the appropriate management (Bloom, 2009). These create disease in the normal ear and alter the environment enough so as to allow for secondary infections to develop (Restrepo, 2013). Table 2 lists the most common primary causes of otitis, as stated by Bloom (2009), Fontaine (2009) and Restrepo (2013).

Table 2 – Common primary causes of otitis externa

þ Canine atopic dermatitis
þ Foreign bodies
þ Parasites
þ Endocrine disease
þ Autoimmune or immune-mediated disease
þ Keratnization disorders

5.3.3. Secondary causes

Not all authors include secondary causes when addressing otitis externa and there seems to be some overlap between these and the so-called predisposing factors. Restrepo (2013) and Moriello (2013) make mention of secondary causes and refer to them as those capable of

creating disease in an already abnormal ear. These causes are of relative ease to eliminate and examples of such are short-listed (Table 3) (Fontaine, 2009; Moriello, 2013; Restrepo, 2013).

Table 3 – Common secondary causes of otitis externa

þ Bacteria	þ Adverse reaction to medication
þ Fungi	þ Physical trauma
þ Overcleaning	þ Yeast overgrowth

5.3.4. Perpetuation

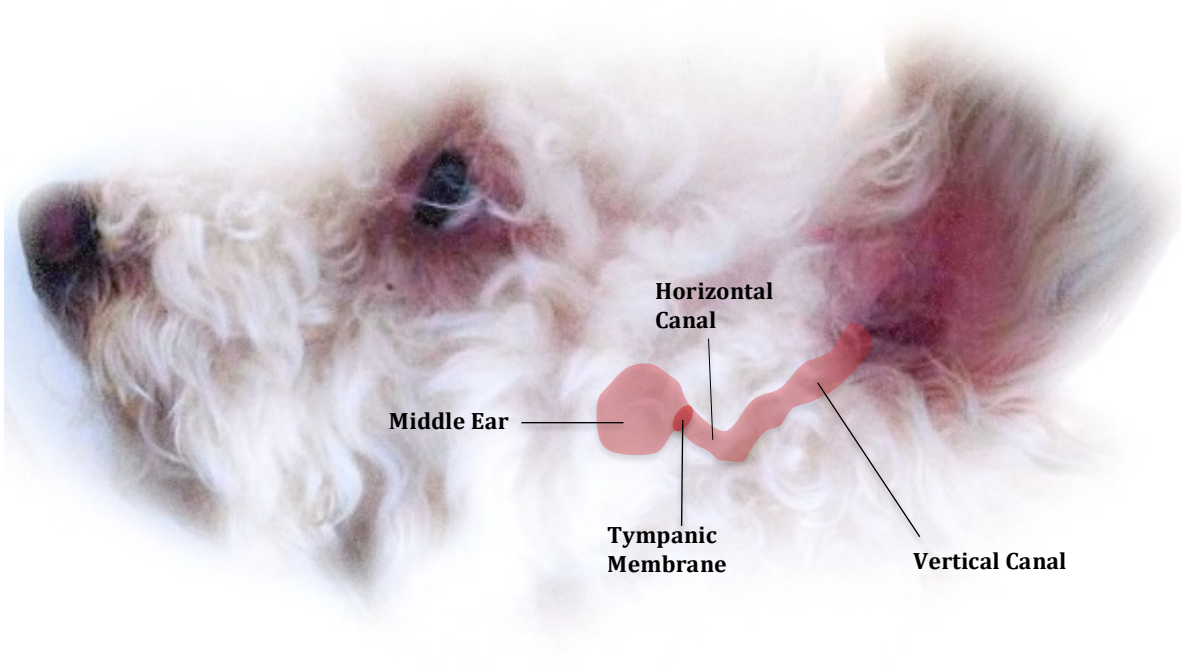
Factors which are classified as perpetuating or predisposing, as addressed previously, encompass elements of the disease itself or of the pet, which can progressively contribute to or promote otitis externa through alteration of structure, function or physiology of the ear canal and its environment (Fontaine, 2009; Restrepo, 2013). In contrast to the predisposing factors, which are present before ear disease develops, perpetuating factors (Table 4) occur due to the inflammation resulting from already existing ear disease (Moriello, 2013). These factors are not capable alone of causing otitis externa but are usually held responsible for the disease when in fact, the problem lies in the primary cause not having been addressed or eliminated. They therefore account for the continuation of ear disease (Bloom, 2009).

Table 4 – Common perpetuating factors of otitis externa

þ Bacteria	þ Fungi/yeast
þ Progressive pathologic changes	þ Otitis media
þ Proliferative changes/altered migration	þ Undertreatment – dose/duration
þ Tympanic rupture	þ Ear canal edema

Interestingly, Bloom (2009) refers to bacteria and yeast as perpetuating factors and does not make mention of secondary causes, while Restrepo (2013) includes such factors in a list of secondary causes, thus suggesting the overlap and classification differences between the categories. Further yet, Bugden (2013) considers microorganisms such as yeasts and bacteria as both predisposing and perpetuating factors.

Fig. 12 - Representation of the canine auricular canal (original source).



The predisposing factors, such as the anatomical conformation (Fig. 12) of long ears, such that they fold and cover the ear canal, create a moist and warm environment, which in itself favors the proliferation of microorganisms (Bugden, 2013; Fontaine, 2009; McQuillan, 2005). The excessive moisture generated by frequent wetting of the canal leads to maceration of the stratum corneum lining the external ear, removing the protective barrier and thus leaving the ear susceptible to opportunistic agents. This humidity may also stimulate the activity of the ceruminous glands, leading to ceruminous otitis externa (Rosser, 2004). There also exists a lack of air ventilation and consequent impaired drying, which contribute to the pathophysiology of otitis (Bugden, 2013; McQuillan, 2005).

5.4. Bacterial and fungal agents

The predominating agents in otitis include *Staphylococcus pseudintermedius*, which can be present in low numbers even in the normal ear and the gram-negative agents *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Klebsiella* spp. and *Escherichia coli* (Moriello, 2013; Rougier, Borell, Pheulpin, Woehrlé & Boisramé, 2005). Rosser (2004) goes on to include more pathogens such as *Corynebacterium* spp. and *Streptococcus* spp. once there has been overcolonization of the external ear canal. *P. aeruginosa* is the most common problematic opportunistic agent associated with chronic or recurrent bacterial otitis externa and has a reputation for its innate resistance to most antimicrobials (Pedersen et al., 2007; Rosser, 2004).

Overcolonization will also lead to isolation of the fungal agent *Malassezia pachydermatis* and, occasionally, of *Candida* spp (Rosser, 2004). Nevertheless, *M. pachydermatis* is also present in low numbers under normal conditions in the external ear canal, as it integrates its normal resident flora (Moriello, 2013; Rosser, 2004).

5.5. Clinical manifestation

The most common clinical presentation of otitis externa includes pruritus, which can be translated into head shaking or ear scratching and malodor. In addition, redness and swelling can be present as a sign of inflammation, as well as pain and discharge of variable nature, in accordance to the type of otitis (Fontaine, 2009; Greek, 2004; McQuillan, 2005; Moriello, 2013; Restrepo, 2013). Hair loss around the ears may also occur and touching the pinna lightly may reveal high temperature or sensitivity. Depending on the gravity of the otitis there may be lesions with scales or crusting. Also, if there is involvement of the middle ear, signs of facial nerve paralysis and head tilt may appear (McQuillan, 2005).

5.6. Diagnosis

A generalized diagnostic approach should be taken and therefore should begin with a full otic and dermatological history and examination. Enquiry should be made regarding concurrent skin disease, age of onset, presence of pruritus, whether there is a seasonality factor, family history and response to any type of therapy previously undergone (Greek, 2004; Restrepo, 2013). The age at which the patient first developed otitis may give insight into underlying causes, for example atopic dermatitis or adverse food reactions, if the first episode took place anywhere in between 6 months to 5 years of age. If the otitis is a seasonal occurrence, then this could lead to possible atopic dermatitis as the subjacent cause. Furthermore, ascertaining whether the episode is a first, a recurrence or an unresolved infection can be difficult due to lack of good follow-up and owners´ perception of cure (Bloom, 2009). Dogs with ear disease oftentimes have concurrent skin disease and therefore should undergo a full dermatological examination, which may possibly lead to the primary diagnosis (Bloom, 2009; Greek, 2004; Restrepo, 2013).

An otic examination should be performed systematically in order to cover all areas and avoid missing alterations (Bloom, 2009; Greek, 2004; McQuillan, 2005; Yu, 2013). Gentle palpation of the ear canal can indicate presence of fibrosis or calcification of the cartilaginous structures, as well as being a clear indicator of pain or discomfort, which, if present, could justify sedation for further examination (Bloom, 2009; Restrepo, 2013). The unique curvature of the canine ear requires use of an otoscope for proper examination and alterations such as inflammation, ulceration, proliferative changes and presence of exudate can be detected.

Visualization of the tympanic membrane is also an essential part of the examination, as well as its condition, which in some pathologic states may be abnormal or even ruptured, indicating otitis media (Bloom, 2009; McQuillan, 2005). In the event that it is not possible to properly observe the ear canal, for example due to presence of excessive amounts of discharge, flushing of the canal can be done to clear the path for better visualization. Resorting to use of a video-otoscope can provide greater magnification of the ear canal and tympanic membrane, as well as allow for manipulation of biopsy instruments and catheters for flushing debris if required (Moriello, 2013).

Plant (2009) considers otic cytology to be the most valuable diagnostic test when managing otitis externa. This procedure should be of routine and thus be conducted both upon initial diagnosis and on follow-ups. Bloom (2009) recommends that cytology samples be collected from the ear canal prior to examination with the otoscope, as it may compromise true appearance of the canal, for example by compacting debris into the horizontal canal and consequently covering the tympanic membrane. Samples should be collected with use of cotton swabs, which should then be rolled on a glass slide and stained with modified Wright´s stain for examination of yeasts, bacterial and inflammatory cells. Samples should first be examined at low-power magnification and then under high-power with immersion oil. Count and morphology of bacteria, yeasts, white blood cells and evidence of phagocytosis of microorganisms should be noted (Plant, 2009; McQuillan, 2005; Moriello, 2013; Restrepo, 2013).

Collecting a sample for bacterial culture and susceptibility testing is also appropriate when rod-shaped bacteria are found upon cytology or in cases of poorly responsive infections, as well as when contemplating systemic therapy (Plant, 2009; Restrepo, 2013).

Additional diagnostic tests may be required due to the possibility of existing underlying causes of otitis externa. Implementing a hypoallergenic diet, performing intradermal allergy tests, conducting a skin scraping, skin biopsy, thyroid testing and other blood analyses should be considered rationally with aid of a proper history and dermatological examination (Moriello, 2013; Plant, 2009).

5.7. Treatment

Otitis externa is usually treated empirically, with the choice of antimicrobial agent being based on the clinical examination, usually comprised of otoscopic examination of the ear canal, cytological evaluation and relevant clinical experience (Bugden, 2013). Nevertheless, in the presence of rods or treatment failure, therapy is prescribed accordingly through aid of culture and further analyses. Management usually depends on the identification and control of

all the causes and factors involved in causing the disease, to the maximum extent possible (Restrepo, 2013).

Proper cleaning of the ear canal is also an imperative form of treatment in that it can prepare the ear canal for maximum efficacy of the subsequent therapeutic agents and prevent future recurrences. Bloom (2009) considers this to be the first step in the treatment of otitis externa, as cleaning agents soften and emulsify cerumen and lipids, aiding in removal of debris. Ceruminolytic agents are commonly used during in-hospital ear cleaning and work by surfactant, detergent or bubbling action. These are, however, contraindicated in cases of ruptured tympanic membrane, due to their ototoxicity. For routine cleaning and maintenance at home, ear cleaning or drying agents may be used, which contain an acid with or without associated alcohol. Topical antiseptics may also be used in the actual treatment of otitis and chlorhexidine-containing agents have a broad-spectrum activity against many gram-positive, gram-negative bacteria and fungi (Cole, 2013).

Topical treatment is known to be the foundation of any otitis externa treatment and most of these commercial products are composed of a combination of glucocorticoids and antibacterial and/or antifungal agents in a vehicle base (Bloom, 2009; Cole, 2013; Moriello, 2013). The advantage of using topical therapy lies in the elevated local concentrations which can be achieved, ranging from 100 to 1000 times the plasma level of the agent in question. It is also important to note that when considering culture and sensitivity results to determine a therapeutic regimen, that such indicate only the plasma level of the antimicrobial agent and therefore cannot be considered an accurate indication of topical therapy outcome (Cole, 2013).

Aminoglycosides, for example gentamicin and neomycin, are commonly employed in the topical treatment of otitis and are efficient against gram-positive and gram-negative organisms (Bloom, 2009; Guardabassi, Schwarz & Lloyd, 2004). Where chronic otitis externa is concerned, multi-resistant *P. aeruginosa* is usually involved and the treatment generally includes topical and/or systemic use of fluoroquinolones or ticarcillin (Guardabassi, 2004), as well as Polymyxin B (Bloom, 2009; Greek, 2004; Moriello, 2013). Effectively utilized antifungal agents include Nystatin, clotrimazole 1%, miconazole 1% and ketoconazole 0,1% (Bloom, 2009; Yu, 2013).

When gram-negative organisms are solely or primarily involved in otitis EDTA integrates the treatment plan, as it has direct bactericidal action against these types of bacteria. EDTA-containing products also exist in association with 0,1% ketoconazole in order to target concurrent *Malassezia* spp (Bloom, 2009). Bloom (2009) raises concerns regarding such combinations due to risk of resistance to ketoconazole with prolonged use and their

alkalinizing effect on the ear as opposed to the desired acidification when treating otitis by *Malassezia* spp.

Topical glucocorticoids are a frequent prerequisite to successful otitis externa treatment in that they are antipruritic and anti-inflammatory. They also reduce glandular secretions, pain and swelling, thus aiding the restoration of the normal barrier function of the epithelium of the ear canal. Commonly employed agents range from betamethasone, fluocinolone and dexamethasone, to prednisolone and hydrocortisone (Bloom, 2009; Greek, 2004; Yu, 2013). For allergic patients, for example, whose ears are not infected, only anti-inflammatory products are needed (Greek, 2004).

A study by Hill et al. (2006) which evaluated the prevalence and treatment of dermatological conditions in United Kingdom small animal practices concluded that systemic antibiotics were prescribed in 25% of cases. Systemic antibiotics tend to be employed in otitis if there is evidence of otitis media or if there are severe proliferative changes within the ear canal and failure in response to topical therapy (Bloom, 2009; Greek, 2004; Moriello, 2013). Empirical choices for cocci consist of cephalosporins, amoxicillin-clavulanic acid and clindamycin. For rods, cephalosporins, amoxicillin-clavulanic acid and potentiated sulfonamides can be used, with the reservation of fluoroquinolones for culture-proven resistant gram-negative rods (Bloom, 2009).

Whatever the regimen chosen to treat otitis externa, effective therapy will depend primarily on the performance and compliance of daily topical treatment by owners in their home environment (Boda, Liège & Rème, 2011).

6. ANTIBIOTIC USAGE

6.1. Antibiotics throughout history

As the world enters a so-called “post-antibiotic era” (World Health Organization [WHO]), 2014, p.9) in which antibacterial drugs have been misused and overused to a point of exhaustion, antimicrobial resistance has begun to disseminate at frightening speed. Alexander Fleming himself had given warning of the potential of this resistance mechanism in 1945 and although being considered a normal evolutionary process, the selective pressure exerted upon it served only to accelerate it. The 1970s saw the development of many new and promising antibacterial drugs that were, in fact, very effective, with the last ones being discovered in the 1980s (WHO, 2014).

6.2. Current global scenario

A study by Silver (2011, p.72) speaks of a current "discovery void" in which no distinct class of antibacterial drugs has been discovered, with the last one dating back to 1987. The world now faces three major intertwined obstacles; not only is there a growing threat of the development of new resistant strains of bacteria, but there is also an ongoing battle against the ones already in existence. To further worsen this scenario, there is a heavy deficit of effective alternate treatments (Silver, 2011). It therefore becomes imperative to find such alternative treatments to be able to motivate reduction in antibiotics and thus combat the emerging resistance (Stobberingh & Vandersanden, 2010).

As evidenced in the alarming report by the World Health Organization (2014, p.1), "It is essential to preserve the efficacy of existing drugs through measures to minimize the development and spread of resistance to them, while efforts to develop new treatment options proceed."

6.2.1. Zoonotic potential

Growing urbanization and changes in domestic habits have motivated the canine transition from that of a guard to essentially a companion, with the natural movement of dogs from the outdoors and directly into the household (Guardabassi et al., 2004; Martins, Peleteiro, Correia & Almeida, 2010). According to the American Veterinary Medical Association ([AVMA], 2012) in 2011, six in ten pet owners, or 63.2%, considered their pets as being family members. Based on this perception that pets are actual members of the family, close physical contact occurs much more frequently, including petting and licking. Not only have companion animal numbers substantially increased in modern society but also much more attention has been vested in pet welfare (Guardabassi et al., 2004).

The shared environment of humans and their animal companions makes possible a transfer of resistant bacteria (Pedersen, Pedersen, Jensen, Finster, Jensen & Heuer, 2007). It is already known, for example, that household pets can serve as reservoirs of bacterial species and resistance genes which are clinically relevant to humans, such as MRSA, VRE and multidrug-resistant *Salmonella typhimurium*. However, regarding the former, it should be noted that pets appear to become such reservoirs due to exposure to infected humans, making them unlikely to constitute a primary reservoir and acting instead as a small secondary reservoir (Guardabassi et al., 2004; Weese & van Duijkeren, 2010).

6.2.2. Resistance in the veterinary scenario

It is the very increased attention devoted to animal welfare which also results in a greater use of antimicrobial agents. Small animal medicine, more specifically canine medicine, makes

frequent usage of preparations licensed for human use and other primary compounds that integrate treatment of human infections (Guardabassi et al., 2004). The consequences of antimicrobial use in small animal medicine are not at all different than those of animal production or even human medicine. It is the amount and pattern of use which determine the rate at which resistance develops and spreads in the bacterial population at hand (Guardabassi et al., 2004).

The Genus *Staphylococcus* encompasses various opportunistic pathogens, of which the most clinically relevant in veterinary medicine are the coagulase positive *S. aureus* and *S. pseudintermedius* (Weese & van Duijkeren, 2010). Their ability to acquire resistance to antimicrobials through the presence of the *mec*A gene makes them particularly relevant, with methicillin-resistant *S. aureus* (MRSA) and methicillin-resistant *S. pseudintermedius* (MRSP) having arisen steadfastly.

With particular regard to methicillin-resistant *Staphylococcus aureus* (MRSA), which is notorious as a human pathogen in hospitals and communities, it has now also become a pathogen of animals, though most animals that encounter it have no problems but opportunistic infections may develop (Faires, Traverse, Tater, Pearl & Weese, 2010; Weese & van Duijkeren, 2010). Faires et al. (2010) identified the highest prevalence of MRSA in skin infections, such as pyoderma and ear infections, as in otitis. These results can be justified by the frequent treatment with beta-lactams and fluoroquinolones, respectively, as well as the chronicity factor which can select for the development of drug resistance (Faires et al., 2010). Another study by Öztürk, Avki, Türütoğlu, Yiğitarslan & Sağnak (2010), also found that MRSA was found predominantly in otitis externa and skin infections.

An important difference between MRSA and MRSP is that colonization by the former is uncommon in humans even among those with frequent contact with animals, thus giving it less importance in terms of zoonotic potential (Weese & van Duijkeren, 2010). *S. pseudintermedius* is the predominant staphylococcal pathogen in dogs, though also being considered a normal inhabitant of the skin and mucosae (Weese, Faires, Frank, Reynolds & Battisti, 2012; van Duijkeren et al., 2011). It is thought to be a resident of areas such as the nares and oropharynx to the anal ring of normal dogs, being able to spread from these to other sites throughout the body, with populations dropping with the use of topical antibiotics, thus also indicating a transient nature (Muller, Griffin & Campbell, 2013). As with other transient organisms, it is sometimes associated with opportunistic infections, with skin and ear infections predominating. In addition, MRSP has become an important agent in pyoderma, otitis externa, urinary tract infections, wound and surgical site infections (Rubin & Chirino-

Trejo, 2011; Weese et al., 2012). MRSP isolates prove difficult to address because they are often not only resistant to beta-lactam antibiotics but also to several other antimicrobial drug classes. Further yet, seeing as many MRSP infections involve post-surgical wounds, management would ideally involve means other than antimicrobial drugs (van Duijkeren et al., 2011), thus calling for other alternatives, to which honey use can possibly respond to.

It is also of worth mentioning that although small animal practitioners can count on stronger economic resources to support laboratory analysis and antimicrobial therapy in comparison to large animal veterinarians, numerous impediments also arise. Diagnostic uncertainty, concerns regarding secondary infections and pressure on behalf of owners may all lead to inappropriate use of antimicrobial agents. The latter point regarding owner pressure is of particular interest when justifying the increasing use of broad-spectrum agents in that veterinarians are afraid of possible treatment failure that might arise from use of first-line antimicrobials. Treatment failure is detrimental to pet health and oftentimes discourages owners who are resistant to investing in additional procedures and consultations (Guardabassi et al., 2004).

6.2.3. Resistance with regard to otitis

According to Guardabassi et al. (2004, p. 322), "The most frequent causes of antimicrobial treatment in dogs and cats are skin and wound infections, otitis externa, respiratory infections, and urinary tract infections". Otitis externa, along with pyoderma are considered to be some of the canine infections which often end up requiring repeated and prolonged treatment regimens (Guardabassi et al., 2004). Pedersen et al. (2007) reported on the occurrence of antimicrobial resistance in bacteria obtained from diagnostic samples in dogs. 5 out of the 6 bacterial species evaluated in the study for being among the most frequently isolated from various infectious conditions in dogs were present in the ears. Such bacteria included *S. pseudintermedius* and *P. aeruginosa* isolates, with the former having been exclusively derived from cases of otitis externa (Pedersen et al., 2007). *S. pseudintermedius* isolates presented highest resistance for penicillin (60,2% of isolates), followed by macrolides and tetracycline, with total susceptibility to amoxicillin with clavulanic acid. As for *P. aeruginosa* all isolates were resistant to ampicillin, amoxicillin with clavulanic acid, cefalotin, clindamycin and erythromycin, most were also resistant to chloramphenicol, spectinomycin, tetracycline and sulphonamides with trimethoprim. Only 35,9% were resistant to enrofloxacin and 15.4% to gentamicin, with only one isolate being resistant to colistin (Pedersen et al. 2007). Such results may be attributed to the fact that otitis externa tends to become chronic and is

subjected to long periods of treatment, thereby selecting for antimicrobial resistance (Pederesen et al., 2007).

III. L-MESITRAN® IN THE MANAGEMENT OF CANINE OTITIS EXTERNA – A PILOT STUDY

1. OBJECTIVES OF THE STUDY

The present study was composed of primary and secondary objectives.

Primary

1. Assess the therapeutic efficacy of L-Mesitran® Soft in the management of canine otitis externa of bacterial and/or fungal (*Malassezia* spp.) etiology.

Secondary

2. Develop an effective alternative to conventional otitis externa treatments;
3. Promote the reduction in use of and prevent resistance development to antibiotic agents.

2. MATERIALS AND METHODS

2.1. Study Design

This was a prospective, controlled clinical trial.

As with any product applied within the ear there was concern regarding ototoxicity, due to the very thin barrier which separates the outer ear from the middle ear; the tympanic membrane. The commonly used agents in the treatment of otitis externa do not possess labelled indication for use in otitis media, i.e. cases in which the tympanum is not intact. Such products are thus frequently used in an off-label manner.

A study by Aron, Akinpelu, Gasbarrino & Daniel (2013) on the safety of transtympanic application of honey in chinchillas concluded that a 4% concentration of manuka honey appeared to be non-toxic to cochlear cells in the middle ear, while a 50% concentration showed severe toxicity.

Evaluation of the transtympanic effect of L-Mesitran® would require additional elaborate procedures and experimental models, which surpassed the intents of the present study. Therefore in order to guarantee maximum safety and avoid potential risk it was deemed prudent to include only subjects with a diagnosis of otitis externa and confirmed visible and intact tympanic membrane.

2.2. Participants

The animals were selected with basis on the following inclusion criterion:

Table 5 – Inclusion criterion for dogs

þ Over 4 months of age;
þ General good health;
þ Confirmed diagnosis of bacterial/fungal/mixed otitis externa.

Dogs eligible were of any sex or breed and the diagnosis of otitis externa was made with basis on clinical signs and cytological evaluation of ear swabs. The diagnosis could be made during the initial presentation; first-time episode or be due to a relapse of a previous episode; recurrent otitis. In cases of bilateral otitis externa both ears were considered for evaluation, with each ear equaling one experimental unit. Signed consent was obtained from the owners of each participant prior to their inclusion in the study.

Dogs were not eligible to participate in this study based on the following exclusion criterion:

Table 6 – Exclusion criterion for dogs

þ Topical or systemic antifungal/antibiotic/corticoid/cyclosporine treatment in the 7 days prior to commencing trial;
þ Long-acting injectable glucocorticoid treatment in the 3 months prior to commencing trial
þ Ears cleaned with an antiseptic product on baseline;
þ Negative microbial cytology;
þ Evidence of ruptured tympanic membrane – as confirmed through video otoscope during initial presentation;
þ Pregnant or lactating females;
þ Evidence of associated pyoderma;
þ Parasitic otitis;
þ Otitis due to foreign body;
þ Advanced stages of proliferative or occlusive otitis.

2.3. Treatments

For the realization of this study in particular L-Mesitran® Soft was chosen due to its more gentle characteristics and taking into account the fragile environment in which it would be applied, the ear. L-Mesitran® Soft also showed the highest antibacterial potential after 24 hours at the lowest dilution in the previously mentioned comparative study (Stobberingh & Vandersanden, 2010). Seeing as only otitis externa was assessed and selected patients had previous confirmation of intact tympanic membrane through use of a video otoscope, concerns regarding ototoxicity in the middle ear did not apply.

2.3.1. Treatment presentation

The L-Mesitran® Soft was generously provided by Triticum® (Netherlands) for this study. The content was divided accordingly among the enrolled patients into 2 ml syringes so that owners could administer 1 ml daily *per* ear. Owners with dogs presenting bilateral otitis were asked to keep separate half of the treatments for each ear, avoiding the mixing of syringes and risk of cross contamination. Owners whose pets presented with unilateral otitis were given half of the quantity of the bilateral weekly treatments.

2.4. Phase I: Tolerance study

2.4.1. Comfort assessment

A preliminary study was conducted prior to the clinical trial in order to assess patient tolerance to the L-Mesitran® Soft in the ear canal, due to its honey content and the possibility of discomfort.

Upon otoscopic evaluation and confirmation of integral tympanic membrane, L-Mesitran® Soft was applied to the ears of 10 healthy, privately owned dogs of voluntary owners, composed of hospital staff and friends. It was established, through previous clinical experience with common otitis treatments, that normal reactions to product application would include head shaking momentarily, as with any substance administration directly into the ear canal, after which total normalcy would be re-established.

The dogs remained under observation for 15 minutes in order to assess their reaction to product administration, whether there was any discomfort and if so, to what degree. Such findings were assessed through use of a short form of the Glasgow Composite Measure Pain Scale (University of Glasgow, 2008).

The importance of this confirmation lied in that one of the aims of this trial was to provide an efficient and convenient management of otitis, to which owners could easily comply with, whilst simultaneously avoiding their pets´ discomfort.

2.4.2. Glycemia assessment

For this study dogs´ glycemic levels were also measured, both before and after L-Mesitran® administration, in order to guarantee maintenance of normoglycemia during the clinical trial phase. As in the comfort assessment, the same dogs of volunteering staff and friends were observed. Although this aspect has not proven to be of concern in diabetic human patients during previous trials with the product, as it did not raise blood glucose levels (L-Mesitran®, 2009) and it would make sense that the same results apply to dogs, it was advantageous to obtain confirmation in the veterinary field. Nevertheless, if a raise in glycemic levels were to be observed, diabetic dogs would then become part of the exclusion criterion.

2.5. Phase II: Clinical Study

The L-Mesitran® was administered by the owners in a home environment, once daily (ex. night). The product was administered initially in the clinic environment to demonstrate the correct technique and thereafter, by the owners until the pre-established clinical cure or during 21 days. Immediately after application the ear was massaged momentarily to ensure uniform distribution throughout the external ear canal.

Owners were asked to bring their dogs for a weekly evaluation at the veterinary hospital so that a clinical and cytological evaluation could be made by the clinician, in addition to a subjective evaluation of improvement by the owner with basis on a 10 cm visual analog scale. Furthermore, in order to obtain a general sense of owner experience a 5 question enquiry was provided to each owner upon conclusion of their dogs´ trial and regardless of duration.

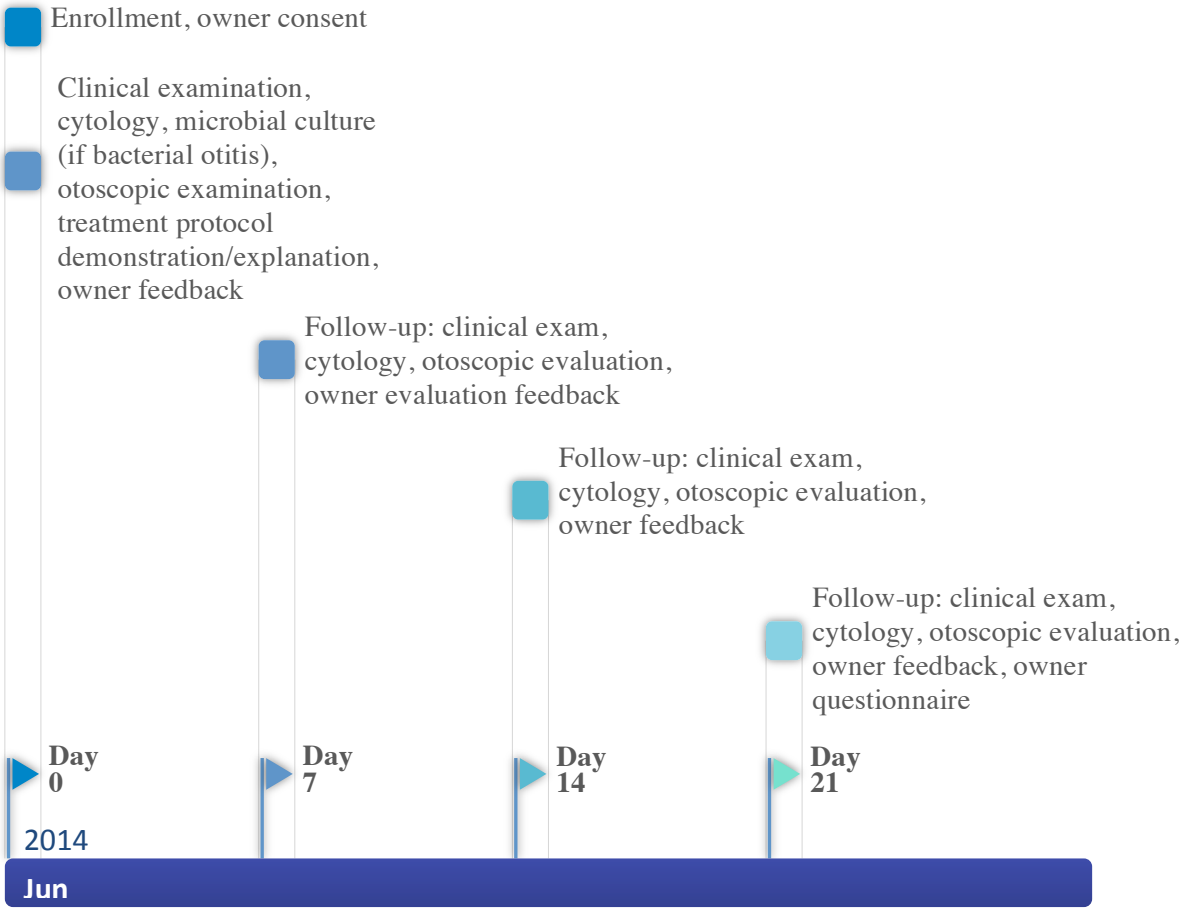
While the trial period for the purpose of this study ended after 3 weeks alternative treatment would go on as required until each dog achieved clinical cure.

2.5.1. Schedule

After having been selected for the trial with basis on confirmed diagnosis of otitis externa of mixed, bacterial or fungal etiology the dogs were considered to be on their baseline (D0), during which the treatment protocol was explained to the owners. From baseline the dogs were scheduled for visits once a week. During each visit a clinical examination was conducted to evaluate 4 pre-established clinical signs, ear samples were collected for cytology and owners assessed pruritus on a visual analog scale.

The end point was considered to be when either clinical cure was achieved during any of the visits or on day 21 (3 weeks). Furthermore, if at any time during the duration of trial the participant required treatment other than that which was assigned and/or if the owner expressed a wish to withdraw their dog from the trial, such incidences were also considered end points and were recorded.

Fig. 14 - Example of 21-day treatment schedule *per* enrolled canine in the clinical trial.



2.5.2. Clinical examination

Nuttal & Bensignor (2014) recently outlined the need for development of a validated, objective clinical score for classifying otitis externa, as a lack thereof makes it difficult to compare clinical trials. In contrast to other pathologies, for example canine atopic dermatitis, which can be objectively assessed through use of the Canine Atopic Dermatitis Extent and Severity Index-04 (CADESI-04), previously used scores for otitis externa are of such a variety that they cannot be uniformly applied. Addressing this pending issue and with basis on previous studies by Rigaut, Sanquer, Maynard & Rème (2011) and Boda et al. (2011), among other studies utilizing varied clinical scores, Nuttal & Bensignor (2014) demonstrated that the 0-3 Otitis Index Score (OTIS3) was considered most suitable for further validation and was thus evaluated in more detail.

In semblance to the previous study, the affected dogs in this trial were divided into erythroceruminous or suppurative otitis based on the clinical signs and type of exudate at presentation. The tympanic membrane also had to be visualized, as its non-visualization, rupture or absence was a strict exclusion criterion. The final result of the study by Nuttal &

Bensignor, (2014) was the identification of a scale which assessed erythema, edema/swelling, erosion/ulceration and exudate of both the horizontal and vertical ear canals, from 0-3, giving a total score of 0-12 (Table 7). It was considered that such a scale could be used to evaluate clinical outcomes in canine otitis externa and in this sense allow for outcome comparisons. The OTIS3 was therefore used in this clinical trial and the numerical scores were attributed to the pre-determined clinical signs. During each visit the ears were examined and evaluated by the same investigator.

Table 7 – Clinical signs and respective scores (according to Nuttal & Bensignor, 2014).

w Erythema	0 = absent; 1 = slight; 2 = moderate; 3 = severe.
w Edema/swelling	0 = absent; 1 = slight; 2 = moderate; 3 = severe.
w Erosion/ulceration	0 = absent; 1 = slight; 2 = moderate; 3 = severe.
w Exudate	0 = absent; 1 = slight; 2 = moderate; 3 = severe.

Nuttal & Bensignor (2014) also found that the most reliable cut-off score which differentiated clinically affected ears from healthy ears was ≥ 4. The total clinical score was calculated through the summation of the individual scores of each clinical sign at each weekly visit. The baseline clinical score would provide a measure of efficacy at the end of the study. Also in keeping with the previous study (Nuttal & Bensignor, 2014), a score of ≤ 3, in this case by the final possible visit on day 21, would be considered a clinical success. Every adverse effect, if at any time observed, had to be reported.

2.5.3. Cytological examination

A swab sample of the external ear canal was obtained at each visit in order to perform cytology. The content was smeared as a thin layer on a glass slide and the DiffQuik procedure was used. The preparations were first fixated and then stained with 10 dips for 1 second at a time for each of the two stains. The slides were then rinsed under tap water and allowed to dry for inspection first under low power and then under oil immersion for detailed view (Royal Veterinary College, 2009).

A study by Ginel, Lucena, Rodriguez & Ortega (2002) sought out to establish a quantitative cytological reference range and correlate it with clinical signs of dogs and cats. In a general sense, with regard to *M. pachydermatis*, mean counts *per* high-power dry field of ≤ 2 yeast cells *per* field in the dog were considered normal, whereas ≥ 5 yeast cells *per* field were abnormally increased. For bacteria, mean counts *per* high-power dry field of ≤ 5 bacteria *per* field in the dog would be normal, with ≥ 25 bacteria *per* field being abnormal (Ginel et al.,

2002). Regardless of the numbers and their high rate of specificity however, the pathogenic role of the organisms would always involve other clinical criteria (Ginel et al., 2002).

2.5.4. Antimicrobial culture, susceptibility testing & biocidal activity testing

In cases of bacterial and/or mixed otitis a sample for bacteriological culture was obtained from each affected external ear canal, prior to cleaning, with a sterile swab, stored appropriately and sent for culture. Isolation and characterization as well as determination of the minimum inhibitory concentrations of the agents so as to investigate presence of antibiotic resistance were conducted through the microdilution method in accordance with 2013 CLSI VET01-S2 norms.

The cultured bacterial isolates were all tested against L-Mesitran® Soft in order to evaluate whether *in vitro* biocidal activity existed. The quantitative assay was conducted in accordance with the NF EN 1040 European Norm. Both biocidal activity testing and culture and susceptibility testing was conducted by the Laboratory of Resistance to Antibiotics and Biocides – FMV.

2.5.5. Sample Size

For the present study 15 animals were considered. To further maximize efficiency, the ear was considered an experimental unit, thus yielding 2 units *per* dog in cases of bilateral otitis.

2.5.6. Efficacy analysis and outcome measurements

Participants were considered in the efficacy analysis if follow-up data was available beyond the first visit, if there were no major deviations to treatment regimens and planned visits and if no conflicting treatments were administered concurrently for an unrelated disease condition. Dogs that were withdrawn from the trial for treatment failure or that required any rescue therapy for the ear condition were included in the efficacy analysis. All animals with any follow-up data after the first visit were considered in the safety analysis.

The main efficacy criterion or main measure of the success of the trial was a clinical score of ≤ 3 until the termination of the trial, or day 21. This followed the notion that clinical resolution of otitis externa is considered a more common indicator of treatment success than microbiological cure for most owners and clinicians in general practice (Grandemange, Pillet, Roy & Woehrlé, 2013). Other efficacy parameters consisted of reduction of the total clinical score and cytology progression from baseline during the weekly visits.

2.5.7. Owner feedback

A secondary outcome measurement was also considered through the participating owners´ subjective evaluation of pruritus based on a 10 cm horizontal visual analog scale (VAS) upon

each visit. They indicated the intensity of pruritus which they believed their animal to have by drawing a short line at the point corresponding to the presumed level of pruritus. The scale at the left edge corresponded to "not itchy" and the right edge "very itchy" (Nuttal & Bensignor, 2014). This allowed for a progressive notion of improvement and comfort of the dogs throughout the treatment, serving as valuable input for the trial in that it gives a sense of the effect of the treatment in the animals´ home environments.

Another outcome of success was considered to be owners overall experience throughout the duration of the trial, by means of a simple 5-question feedback questionnaire (Annex I) at the end of the trial. Though subjective, it was critical in revealing pros and cons of this study, thus providing possible means of improvement, as well as in determining whether continuing studies would be of success, as it reflects, in a sense, owner compliance.

Initiative was also taken to establish monthly contact with owners after having finished treatments in order to assess the duration of clinical cure.

2.5.8. Withdrawal & Clinical failure

Participating owners were entitled to withdraw their animal from the trial at any moment in time and for any reason.

Clinical failure was defined as persistence of symptoms and signs of otitis externa beyond the pre-established time frame of 21 days, as well as a score of ≤ 3, thus justifying the need for additional therapy.

2.5.9. Statistical Analysis

Using R (The R Foundation for Statistical Computing®), the Kaplan-Meier method was applied to the survival analysis evaluating both probability of clinical cure over time, as well as probability of clinical and cytological cures over time. The Repeated Measures ANOVA statistical test in addition to a post-hoc test with Holm´s correction was used to evaluate the clinical scores for each day. Progressive owner evaluation of visual analog scale was also analyzed through the Repeated Measures ANOVA test.

3. RESULTS

3.1. Phase I Results

3.1.1. Comfort assessment

The 10 volunteer dogs were considered to be in good health after a general clinical examination and had no previous history of illness. A Short Form of the Glasgow Composite Measure Pain Scale (University of Glasgow, 2008) (Annex II) was used to assess whether

there was any discomfort associated with the application of L-Mesitran® Soft in the ear canal. The highest total score representative of the maximum level of discomfort was 24 and the lowest score was 0, representing no discomfort whatsoever. Six simple parameters were observed and a numeric value was attributed to each, with an increase in value representing increased discomfort. Based on each of the 6 parameters assessed by this scale all of the scores obtained for each dog totaled 0, thus demonstrating a 100% absence of discomfort. In this sense the administration of L-Mesitran® Soft proved to be at least as tolerable by dogs as any other commonly prescribed otitis treatment.

3.1.2. Glycemia assessment

The 10 volunteer dogs were deemed healthy and none were diabetic. Blood glucose levels were measured at a starting point, with no product having been applied to the ear canal. Results for all dogs were in conformity with normal glycemic levels. Next, 1 ml of L-Mesitran® Soft was applied to each ear canal, so as to simulate treatment for the patients enrolled in the clinical trial. After 20-30 minutes blood sugar levels were measured once more to register any alterations, none of which occurred in all tested dogs. Such results are therefore in accordance with those of human diabetic trials, in which L-Mesitran® usage does not interfere with blood glucose levels and can thus be safely employed.

3.2. Phase II Results

3.2.1. Animals included in the study

The 15 animals included in the study fulfilled the inclusion and exclusion criterion pre-established for this study and were of varying ages and breeds (Annex III). 4 of the 15 enrolled dogs presented with unilateral otitis, thus totaling 26 ears or experimental units evaluated throughout the trial. A number was attributed to each dog in order of enrollment in the study, such that the same number corresponds to the same dog in the following tables in this research.

The enrolled animals came from a mixed home environment and had diverse histories regarding otitis externa. Owners were asked whether their pets were kept indoors, outdoors or both, whether they had other pets in the same household and whether their dogs had the habit of going swimming (Annex IV). With regard to otitis, the number of dogs with their first episode of otitis *versus* dogs that had suffered from recurrent episodes was close to half, with 7 dogs in the former group and 8 in the latter (Annex V).

As mentioned in the review of literature, the canine ear is more predisposed to acquiring otitis, with certain breeds and anatomical types more so than others. For the interest of this study the participating dogs´ ear types were recorded, as were the associated types of otitis (bacterial/fungal/mixed) and their respective locations (bilateral/unilateral) (Annex V).

3.2.2. Clinical Progression

Each patient´s weekly scores were kept for comparison at the end of the trial. Some patients required less than 21 days of treatment, with 3 patients requiring only 7 days to reach both clinical and cytological cure. The obtained clinical scores are depicted in Table 8.

Table 8 – Weekly clinical scores per ear for 26 ears

Animal	Type	Day 0	Day 7	Day 14	Day 21
1	B	6	5	2	1
	B	6	4	2	1
2	F	5	4	2	1
	F	7	4	2	1
3	M	4	0	0	-
	M	4	0	0	-
4	B	5	3	1	-
	B	10	5	1	-
5	M	6	1	-	-
6	M	4	2	1	-
	M	6	3	1	-
7	F	4	1	1	0
8	F	4	1	1	1
	F	6	1	1	1
9	B	7	4	4	4
	B	4	3	2	2
10	F	5	3	2	1
	F	4	3	1	1
11	F	8	3	2	2
	F	7	3	2	1
12	F	4	0	-	-
13	F	6	2	1	1
	F	4	2	1	1
14	F	8	5	4	-
15	M	4	0	-	-
	M	4	0	-	-

F – Fungal. B – Bacterial. M – Mixed.

Of the 15 dogs (26 ears) evaluated, one needed alternative treatment after concluding the 21-day trial due to persisting abnormal numbers (Ginel et al., 2002) of cocci and rods on

cytology, as well as a clinical score of 4. One other dog was voluntarily withdrawn by its owner due to apparent difficulty in administering the treatment. At the point of withdrawal on day 14, this dog presented a score of 4 and abnormal cytology.

3.2.3. Statistical Analysis

Survival analysis was useful in this study as it considers time as the variable of interest until an event occurs and also addresses patient follow-ups, which was appropriate for the trial at hand (Singh & Mukhopadhvav, 2011). Using R (The R Foundation for Statistical Computing®), the Kaplan-Meier method was applied to the survival analysis evaluating the probability of clinical cure over time (Fig. 15). 70% of enrolled dogs achieved clinical cure between days 7 to 14 and over 90% on day 21 with a confidence interval of 95%.

The same method was applied to the survival analysis of the probability of clinical and cytological cures over time (Fig.16). By day 7, 20% of dogs had obtained both clinical and cytological cures.

Fig. 15 - Survival analysis of probability of clinical cure over time

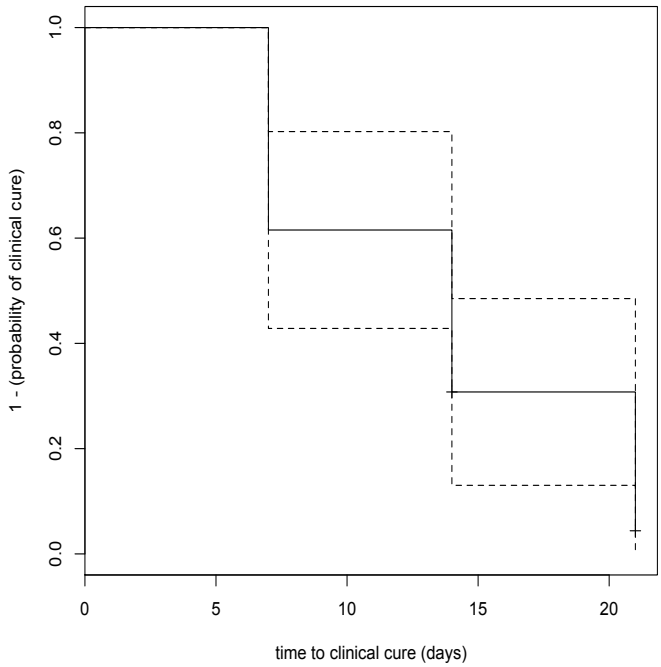
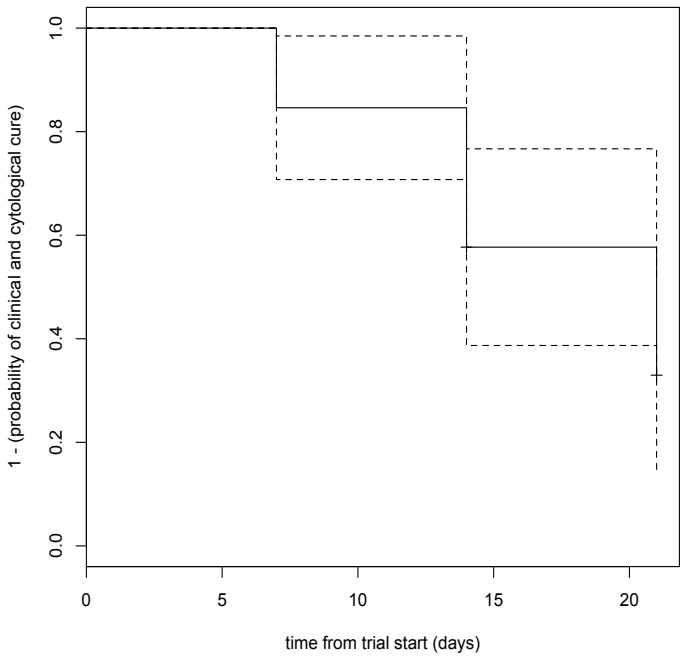


Fig. 16 - Survival analysis of probability of clinical and cytological cure over time



There was a decrease in clinical scores throughout the trial duration (Fig. 17) ($p < 0,001$). The box plot depicting the owner VAS scores (Fig. 18) shows the decreasing values in time ($p < 0,05$), bearing in mind that on day 21 many of the dogs had already achieved cure and were no longer enrolled in the study, thus the respective absence of scores and corresponding results.

Fig. 17 - Box plot evaluating clinical score decrease.

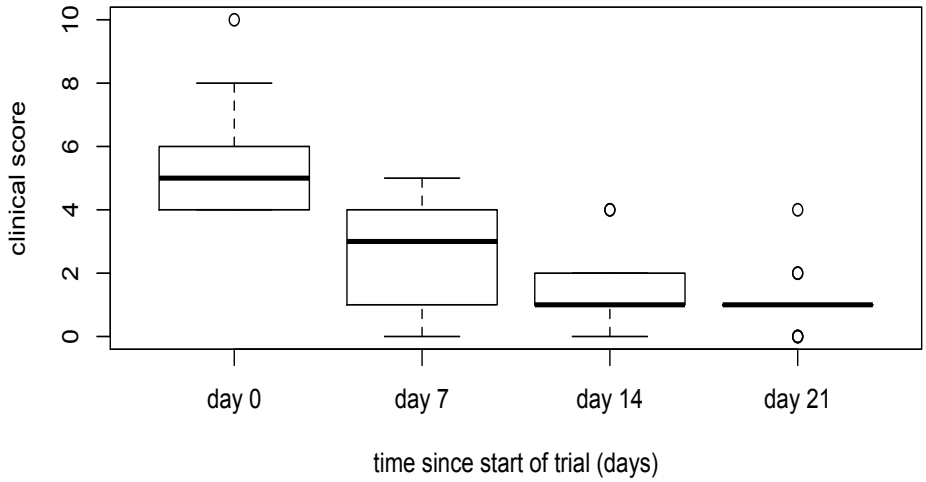
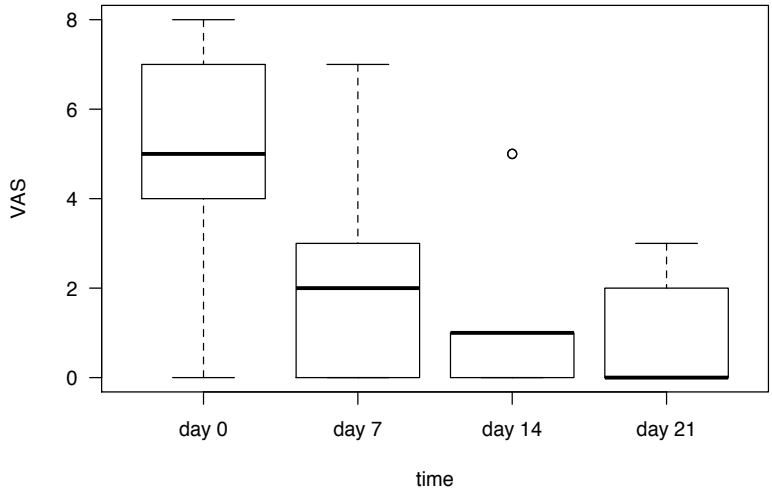


Fig. 18 - Box plot evaluating owner VAS score decrease.

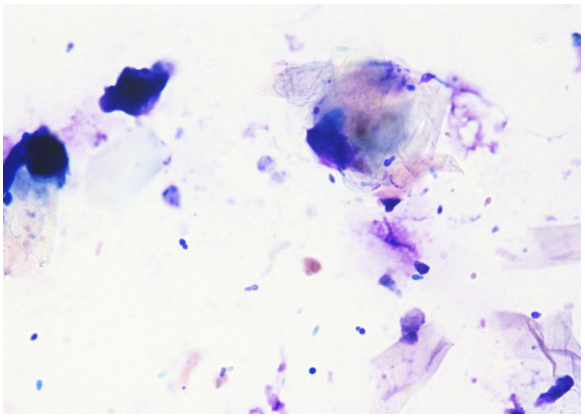


3.2.4. Cytological progression

100% of the enrolled dogs demonstrated significant decreases in numbers of the microorganisms involved in their respective type of otitis (Fig. 19-22). As addressed in the review of literature, the antimicrobial properties of honey have been shown to encompass fungi and bacteria, which was re-confirmed during this trial.

Of the 26 affected ears, a total of 8 ears had not presented the previously established normal numbers of yeasts or bacteria at the end of the treatment (Ginel et al., 2002). Most dogs required longer time periods to achieve cytological cure in comparison with the time they took to achieve clinical cure. Nevertheless, decreasing values were a global constant.

Fig. 19 - *Malassezia* sp. - Day 0 (x400 amplif.).**Fig. 20** - Improvement – Day 21 (x400 amplif.).



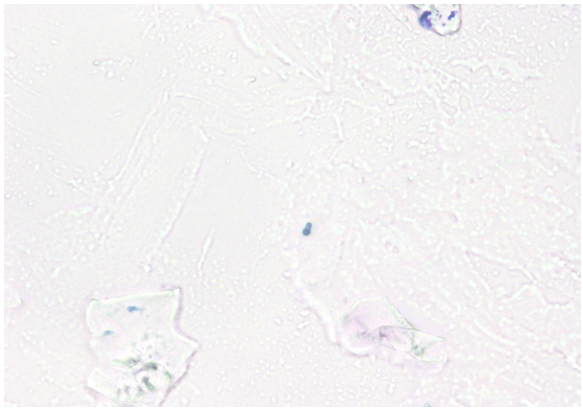
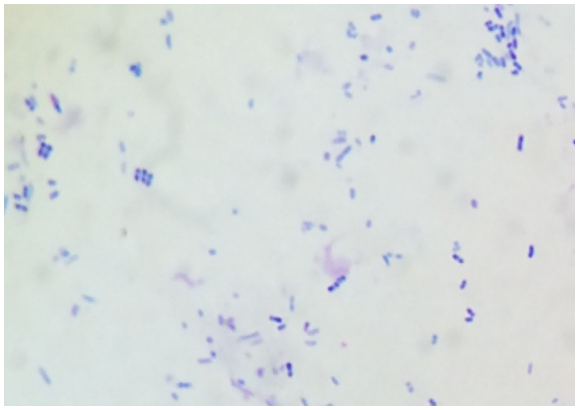
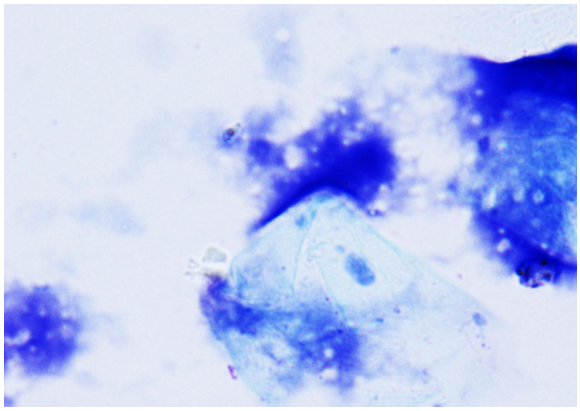


Fig. 21 - Rods & cocci-Day 0 (x1000 amplif.). **Fig. 22** - Improvement-Day 21 (x1000 amplif.).





3.2.5. Antimicrobial culture, susceptibility testing and biocidal activity testing

Isolation, characterization and respective resistance profiles revealed diversified bacteria, all of which are considered to be possible secondary causes of otitis externa (Miller et al., 2013). There was a wide range of agents (Table 9) from common and susceptible *S. pseudintermedius*, *Klebsiella pneumoniae* to highly resistant *Enterococcus faecalis* and 2 findings of the emerging MRSP.

Table 9 – Antimicrobial culture & susceptibility

Antibiotics	**Bacterial isolates**								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Fusidic acid	S	S	S	S	-	-	S	S	S
Amoxicillin/Clavulanic acid	S	R	R	S	R	R	S	R	R
Ampicillin	R	R	R	R	R	R	R	R	R
Cefalotin	-	-	-	-	S	R	S	-	-
Cefotaxime	-	-	-	-	S	R	I	-	-
Clindamycin	S	R	R	S	-	-	-	S	S
Enrofloxacin	S	R	S	S	S	I	S	S	S
Erythromycin	S	R	I	S	-	-	-	I	I
Oxacillin	S	-	S	S	-	-	-	R	R
Gentamicin	S	R	S	S	S	S	S	S	S
Penicillin	R	R	R	R	-	-	-	R	R
Sulfamethoxazole/Trimethoprim	S	S	S	S	S	S	S	S	S
Tetracycline	S	R	S	S	S	I	T	R	R
Chloramphenicol	-	-	-	-	-	-	-	S	S
Florfenicol	-	-	-	-	-	-	-	S	S
Amicacin	-	-	-	-	-	-	-	S	S

1 – *S. pseudintermedius*. 2 – *E. faecalis*. 3 – *S. pseudintermedius*. 4 – *S. pseudintermedius*. 5 – *K. pneumoniae*. 6 – *Enterobacter cloacae*. 7 – *Pseudomonas* spp. 8 – MRSP. 9 – MRSP. S – Susceptible. I – Intermediate. R – Resistant.

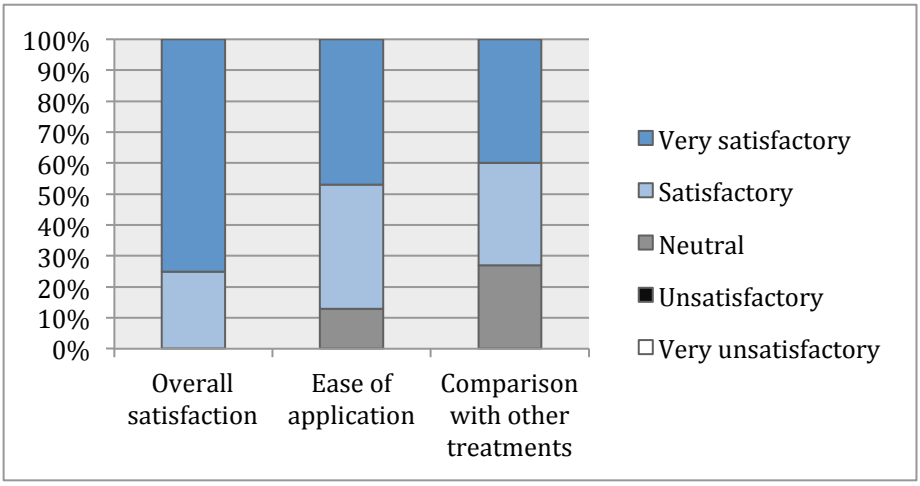
L-Mesitran® Soft demonstrated clear biocidal activity within the first five minutes of contact against all bacterial isolates.

3.2.6. Owner feedback

During each weekly visit, in addition to enquiring about general condition and progress owners were asked to grade the pruritus level which they believed their dogs to have, through use of the VAS. All of the participating owners progressively lowered their grading on the scale during the visits.

On each dog´s last visit a simple 5-question survey was given to owners in order to obtain their final thoughts regarding overall satisfaction with the treatment, ease of use, comparison with other previously utilized treatments in cases of recurrent otitis, whether they would use this treatment again if necessary and finally whether they would recommend it to other pet owners. The totality of owners was satisfied with the treatment itself, with 75% having considered it very satisfactory and the remaining 25% considering it satisfactory, with zero having considered it unsatisfactory or very unsatisfactory (Fig. 23). In addition, 100% of owners answered that they would repeat the treatment as well as recommend it to other owners.

Fig. 23 - Owner survey results.



3.2.7. Follow-up

As it was of interest to obtain a general sense of the duration of the clinical cure of the patients involved in the study, an attempt was made monthly in order to keep in touch with the owners. All of the owners, excluding the 2 cases, one of clinical failure and another of withdrawal, which were not included in this evaluation, kept contact up to 1 month after

ending treatment, at which point none of the dogs had presented relapses. Shortly after 1 month, 1 dog presented with signs of otitis and at 2 months, so did 2 others. Another 2 dogs were also lost to follow-up due to the impossibility of contacting them. The remaining 8 dogs maintained clinical normalcy between 3 to 5 months, with 5 months being the maximum possible time frame during which contact was kept with owners prior to publishing this study. Therefore likelihood that this period will be even greater for some dogs should be taken into consideration. Finally and remarkably, the dog that presented with otitis with MRSP involvement was also included in this last group, with owners reporting total normalcy at 5 months.

4. DISCUSSION

4.1. Overall evaluation of L-Mesitran® in the treatment of otitis externa

The use of L-Mesitran® Soft was globally effective in managing otitis externa and yielded extremely positive results, confirming existing literature regarding the successful use of honey in medicine. Quick onset of clinical and cytological improvement, positive owner evaluations of progression, in addition to culture results proved highly encouraging. More specifically regarding the former aspect, enormous significance was obtained through the fact that treatment was successful towards types of bacteria demonstrating high levels of resistance. In addition, follow-up results were remarkable, as over half of the dogs enrolled had maintained clinical well-being in many of the following months after the trial.

4.1.1. Clinical and cytological progression

3 out of 15 cases of otitis were quickly resolved in only 1 week due to achievement of clinical cure (score ≤ 3) as well as cytological cure. 5 out of 15 dogs were asked to return on remaining 7-day intervals even after having achieved a clinical score of ≤ 3, due to irregular cytology results. Nevertheless, it is important to note that the owners of these 5 dogs did not notice any signs of discomfort or of otitis, which had led them to seek medical care to begin with. By 21 days and excluding 1 withdrawal at day 14, 4 out of 15 dogs did not present cytological cure but had obtained a clinical score of ≤ 3 and demonstrated constantly decreasing numbers in cytology, with value assessment based on Ginel et al. (2002). It can be inferred, due to the cytological progression of the dogs, that had the trial period been greater than 21 days, they would most likely have achieved cytological cure. These dogs were clinically normal and had returned to their regular habits, with owners reporting no discomfort, thus eliminating the need for further treatment. The remaining dogs achieved both clinical and cytological cure by day 21. In a global sense, a corresponding decrease in the

number of microorganisms upon cytological evaluation was observed, though it occurred at a slower rate when compared to the quick attenuation of clinical signs.

4.1.2. Significance of cytological results

Where cytology is concerned there has been much discrepancy between authors regarding what should be considered normal or abnormal in samples from the external ear. Yeast population, for example, is difficult to correlate with clinical signs due to the multifactorial origin of otitis externa, as well as the fact that many dogs with the condition do not present an elevated number of yeasts (Ginel et al., 2002). As Ginel et al. (2002) found, there is no clear cut-off between higher than normal numbers and pathogenically significant bacterial overgrowth. Increased bacterial populations are frequently considered as a perpetuating factor as opposed to a primary cause. This secondary role and the nature of otitis externa may justify the fact that in the study (Ginel et al., 2002) as many as 50% of dogs with otitis externa did not have increased bacterial counts. On the other hand, some samples from dogs with ceruminous otitis presented elevated bacteria numbers without any inflammatory cells, thus suggesting secondary colonization, which would seem to correlate well with the present clinical trial. It can be affirmed that both yeast and bacterial numbers decreased progressively throughout the duration of the trial, for all dogs, although at different rates for each case. In this study, with the exception of 1 animal, whose cytology did not reveal significant decrease in bacteria during the last 2 weeks of the trial and thus needed alternative treatment after day 21, all dogs showed noticeable improvement. It is also fitting to recall that although cytology counts decreased with successful treatment in the study by Nutall & Bensignor (2014), they did not adequately differentiate healthy from affected ears, nor identify clinical success.

4.1.3. Significance of microbiological results

Microbial culture results in this trial were a clear reflection of the current scenario which spreads across the veterinary medical field. Various common and susceptible agents were found in the cases of bacterial involvement and out of a small group of 15 dogs, MRSP was detected twice, thus in keeping with the vast and rapid dissemination of the agent. In addition, one isolate of *E. faecalis* with a highly resistant profile was also found in one case of recurrent otitis.

Of the 2 cases of MRSP, one happened to be the case of clinical failure, due to the need for alternative therapy after end of treatment with L-Mesitran®. At 21 days the dog had not obtained the necessary clinical score to be considered cured, nor were cytological numbers representative of such. Although there had been progressive improvement along the weeks and the owner indicated such, further treatment was instilled. Seeing as this was an elder dog

with nearly an entire life´s history of recurrent otitis and had been subjected to numerous treatments, the outcome becomes understandable. On an additional note, contact was established with the owner nearly 2 months after having ended the alternative therapy, at which point otitis externa had returned.

The other isolate of MRSP was obtained from another dog with an almost opposite history in relation to the previous one. This middle-aged dog had never been diagnosed with otitis before and upon presentation had unilateral otitis externa with *Malassezia* sp. and cocci involvement. Only 7 days of treatment with L-Mesitran® were required for resolution. The owner also reported a rapid and significant improvement with regard to pain and discomfort.

MRSP, like susceptible staphylococci, is also an opportunistic agent and colonization does not necessarily lead to disease but when it does, no indication exists that clinical infection is more serious that with *S. pseudintermedius*, though treatment is more difficult (Weese, 2011). Honey has undergone resurgence due to its bactericidal effects against a varied spectrum of bacteria, including staphylococci (Weese, 2011) and the case encountered in this trial has become a strong representative of such, with the added significance of displaying methicillin resistance. Furthermore, with the presence of a highly resistant *E. faecalis*, the treatment with L-Mesitran® also demonstrated success in improving clinical and cytological condition.

Though the clinical component of this trial was in itself an enormous success, the information obtained from the culture results and particularly the fact that such resistant profiles were found, added tremendous value to this study and towards the use of honey in medicine.

4.2. Treatment formulation, administration & owner compliance

Owner enquiry results and conversations during consultations throughout the clinical trial period indicated faults and attributes of the utilized treatment and its formulation. Perhaps the only inconvenient aspect of the L-Mesitran® Soft in this case was its increasing accumulation inside the ear canal and around the outer portion of the ears, forming a sticky layer of honey, which tended to solidify with time. Some owners found it useful to clean the outer ear with cotton balls moistened with water so as to remove this layer while others resorted to full baths at the end of treatment. Also, some owners mentioned improvement in this sense when utilizing an otic cleaning solution containing Tris-EDTA and chlorhexidine (Otodine®) to clean the ears after ending the treatment regimen, which progressively dissolved the remaining honey product.

A study evaluating owner compliance towards topical treatments for otitis externa by Boda et al. (2011) yielded useful results which could be correlated with the issues raised during this study. In the former study (Boda et al., 2011), a 3-5 drops twice daily, 7-day regimen was

compared with a once daily pump application 5-day regimen, with the sole focus being owner compliance. Results for each regimen were substantially different, with only 10% of owners having stated that they were able to verify the exact number of drops administered in the 7-day regimen (Boda et al., 2011).

Boda et al. (2011) reinforced the importance of properly carrying out topical treatment in order to obtain optimum efficacy, though keeping in mind that administration may prove difficult for owners, especially when the auricular condition of their pet is painful and treatment needs to be performed more than once. With regard to frequency, more owners (100%) administering the pump treatment once a day rated the treatment positively, in contrast to owners (78%) administering the drops treatment twice a day.

The results of the study (Boda et al. 2011) emphasized the importance of setting a simplified dosing regimen, coupled with an equally simple method of administration so that owner compliance can be maximized. On this note, during this present trial one dog was considered a dropout, or withdrawal, by choice of the owner due to reported difficulty in administering the product.

With regard to product formulation and as demonstrated by Boda et al. (2011), the L-Mesitran® Soft used in the present trial could have been adapted to be administered via a simple pump system that could reach the ear canal, delivering a single pre-established amount. This would not only guarantee correct dosage but also possibly avoid product accumulation in the external ear, if the cannula penetrated deep enough. As Boda et al. (2011) reiterated in their study, the ease with which medications can be used is an important element in improving patient compliance and thus treatment efficacy.

Concerning product accumulation, this study design could have benefited, for example, from more tailored dosages of L-Mesitran® Soft, taking into consideration each ear canal with regard to shape and size. This would allow for each dog to be prescribed between 0,5 ml – 1 ml, for example, in accordance to its ears, instead of using a constant dose of 1 ml, which did indeed seem to be unnecessary and even excessive for some dogs with smaller ears. Furthermore, adapting the dosage to the particular case would undoubtedly provide a more cost-effective treatment, comparable to or possibly being more advantageous in this aspect than other commonly prescribed treatments.

4.3. Weaknesses of this trial

As discussed previously, the tailored quantity of L-Mesitran® Soft for each dog may have sufficed for results that were equally positive as those obtained in this trial. Furthermore, with the absence of excess product, owners may have had an even more positive experience with

the treatment in that they may not have needed to clean their dogs´ ears so often. Also with regard to customization of the treatment protocol in accordance with each dog´s need, a more flexible schedule would have been beneficial, as opposed to a maximum of 21 days. This was a logical time frame, established for the purposes of this clinical trial and from the results, it makes sense to infer that some cases would have been successfully resolved had they continued with L-Mesitran® Soft for a while longer. In addition, time seemed a small price to pay in exchange for the avoidance of antibiotics, especially in recurrent cases; an opinion which the respective owners all seemed to share.

Due to the consistency and yellowish appearance of the L-Mesitran® Soft being highly similar to that of ear cerumen, it sometimes became difficult to distinguish between the two throughout the treatment with increasing accumulation. Still, there are a number of other commercially available products used to treat otitis externa which pose the same concern regarding appearance, such as Oridermyl® (Vétoquinol®). Nevertheless, in this trial, the other improving clinical signs were also supportive of the absence of cerumen and the honey present in the L-Mesitran® had a distinctly sweetened odor which helped in differentiating it from often malodorous otitis exudate. In keeping with odor, it is worth noting that a significant difference was observed in cases in which malodor was prominent, such as those involving otitis by *Malassezia* spp. For these participants, visits from day 7 onwards revealed absence of any sort of odor, thus suggesting efficacy on behalf of the L-Mesitran® in tackling microorganisms.

4.4. Other considerations

Another important aspect to be considered when evaluating the global outcome of this study is the multifactorial nature of otitis externa, which was already discussed. In a study by Favrot, Steffan, Seewald & Picco (2010), 43% of dogs with canine atopic dermatitis for example, presented initially with an episode of otitis externa. In cases of food allergies, not all body regions are necessarily affected and manifestations can vary greatly. For example, dogs may present with only otitis externa, occasionally affecting only one ear (Jackson, 2009).

No diagnosis other than otitis externa was made during this trial nor were other possible underlying causes explored. Seeing as many of the participants were sent on behalf of other practicing colleagues for the sole purpose of this study, it would be unethical to explore and treat any conditions other than otitis externa. Therefore, this trial solely addressed the clinical and cytological manifestations of otitis externa. Where acute otitis is concerned, there is sometimes the need for systemic therapy so as not to exacerbate pain or pruritus, facilitate treatment by owners and prevent future chronicity. With regard to chronic otitis externa,

which was the case for some of the participants in this trial, there is a necessity for in-depth investigation and correction of all involved factors, from predisposing to primary to perpetuating factors (Bensignor & Forsythe, 2012). Considering the complexity which is oftentimes implicated in what are seemingly harmless cases of otitis, additional and/or long-term treatment may have been required for resolution in such cases. Regardless of underlying cause however, it can be affirmed that honey managed the clinical and cytological manifestations efficiently.

Also relevant to the possibility of there existing underlying factors which were not addressed is the aspect of follow-up after the end of the trial. It is probable that dogs that achieved cure during this trial would eventually present with otitis again without management of the primary causes involved. Though it was not one of the main objectives of this study, the attempt was made to establish contact with the owners in the following weeks of the trial was made. It would be ideal to gather follow-up data from all the animals over a longer time frame in order to evaluate the maintenance duration of the L-Mesitran® treatment, though each case is different. The fact that some dogs presented on baseline with recurrent otitis and others with first episodes did not seem to determine how long they stayed otitis-free, as some chronic cases are currently on their way to the 6th month without any manifestation and other first-timers relapsed after the 3 months.

During the planning and enrollment phase of this clinical trial the study by Nuttal & Bensignor (2014) used to score clinical signs had not yet been published and therefore a list consisting of 9 criteria had been decided upon with basis on 2 previous studies by Rigaut et al. (2011) and Rougier, Borell, Pheulpin, Woehrlé & Boisramé (2005). The former study by Rigaut et al. (2011) was also assessed by Nuttal & Bensignor (2014) to develop the current scale for otitis externa, OTIS3. Seeing as this most recent scale was a meticulous evaluation and adaptation of a number of other previously utilized scales, this trial´s clinical progression evaluation was also adapted, as much as possible, so as to incorporate OTIS3.

To the best of our knowledge and to date there have been no clinical trials assessing the actual efficacy of honey in cases of otitis externa in dogs. As with any scientific experiment and especially considering there were no precedents, there was always room for self-evaluation and improvement.

5. CONCLUSION

The impressive clinical, cytological and microbiological results yielded from this trial are in accordance with the increasing published information regarding honey´s healing properties.

The fact that honey was able to treat otitis externa and maintain the well-being of the dogs in this study even in the face of highly resistant strains of pathogens including MRSP was imperative in the battle against antibiotic resistance and eventual skepticism towards honey use. Although a small step in terms of numbers, these results now justify further, more in-depth and long-term research, so as to draw more conclusions as to whether honey can one day be included in veterinary practice as a common treatment for otitis externa. As ear and skin infections are oftentimes subjected to long periods of antimicrobial treatments and may generate chronicity, the implementation of alternative treatments would most certainly have an impact on the current scenario and act preventatively in terms of future resistance acquisition. Through this pilot study and through numerous already existing accounts of honey´s success, it seems only fit that it earn a place at the top of the list with regard to potential alternatives in medicine.

6. FUTURE PROSPECTS

The present pilot study was successfully able to demonstrate that the use of honey is effective in managing otitis externa in dogs, thus paving the way to continuing studies. The second phase of this study, which will include a significant increase in participants and will compare L-Mesitran® with another commercial product for otitis externa is set to take place in the start of 2015 and has already been approved for support in funding. In addition, cost-effectiveness is an important aspect to explore, as owners of dogs with chronic cases of otitis tend to spend money more often than not. Comparing the costs of honey versus other market products would possibly also impact owner decision as well.

Final thought should be paid to the ever-increasing proximity between man and dog, through which numerous resemblances among the two have arisen. Several authors including Martins et al. (2010) and Marsella & Sousa (2001), with published work regarding the possibility of a canine model in the study of human atopic dermatitis, have opened doors to new opportunities in the human medical field with basis on veterinary models. This clinical trial involving the use of honey for management of canine otitis externa can be extrapolated for future studies, possibly involving humans, as the general concepts are the same and so is the corresponding urgency to combat arising antibiotic resistance.

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ANNEX I

Owner Enquiry: L-Mesitran in the management of canine otitis externa

a. Overall satisfaction with the treatment

1 2 3 4 5

b. Ease of application of the treatment

1 2 3 4 5

c. Comparison with other previously used treatments for the same problem

1 2 3 4 5

d. Would you perform this treatment again?

Yes No

e. Would you recommend this treatment to other pet owners?

Yes No

1 = Very satisfactory
2 = Satisfactory
3 = Neutral
4 =Unsatisactory
5 = Very unsatisfactory

ANNEX II

SHORT FORM OF THE GLASGOW COMPOSITE MEASURE PAIN SCALE

Dog's name _____ **Date** / / **Time**

Hospital Number _____

Procedure or Condition _____

_____

In the sections below please circle the appropriate score in each list and sum these to give the total score

A. Look at dog in Kennel

Is the dog

(i)		(ii)	
Quiet	0	Ignoring any wound or painful area	0
Crying or whimpering	1	Looking at wound or painful area	1
Groaning	2	Licking wound or painful area	2
Screaming	3	Rubbing wound or painful area	3
		Chewing wound or painful area.	4

In the case of spinal, pelvic or multiple limb fractures, or where assistance is required to aid locomotion do not carry out section **B** and proceed to **C**
Please tick if this is the case ☐ then proceed to C

B. Put lead on dog and lead out of the kennel

When the dog rises/walks is it?

(iii)	
Normal	0
Lame	1
Slow or reluctant	2
Stiff	3
It refuses to move	4

C. If it has a wound or painful area including abdomen, apply gentle pressure 2 inches round the site

Does it?

(iv)	
Do nothing	0
Look round	1
Flinch	2
Growl or guard area	3
Snap	4
Cry	5

D. Overall

Is the dog?

(v)	
Happy and content or happy and bouncy	0
Quiet	1
Indifferent or non-responsive to surroundings	2
Nervous or anxious or fearful	3
Depressed or non-responsive to stimulation	4

Is the dog?

(vi)	
Comfortable	0
Unsettled	1
Restless	2
Hunched or tense	3
Rigid	4

Total Score (i+ii+iii+iv+v+vi) = _____

ANNEX III

General characteristics of participants

Animal	Breed	Sex	Age (years)	Weight (kilograms)
1	Crossbreed	M	14	24
2	Crossbreed	M	11	26
3	Crossbreed	M	1	18
4	Golden Retriever	M	8	34
5	Crossbreed	M	8	7
6	Rafeiro Alentejano	M	11	55
7	Crossbreed	M	3	34
8	Crossbreed	M	2	40
9	Basset Hound	M	12	24
10	Serra da Estrela	F	3	34
11	Serra da Estrela	M	2	36
12	Weimaraner	F	1	27
13	Black Labrador	F	4	40
14	Crossbreed	M	12	14
15	Akita Inu	M	1	43

F – Female. M – Male.

ANNEX IV

Relevant Patient Background			
Animal	**Environment**	**Other pets**	**Swimmer**
1	Indoor	No	Yes
2	Indoor	No	No
3	Indoor	No	No
4	Indoor	No	No
5	Indoor	No	No
6	Outdoor	Yes	No
7	Indoor	No	No
8	Indoor/Outdoor	No	No
9	Indoor	No	No
10	Indoor/Outdoor	Yes	No
11	Indoor/Outdoor	Yes	No
12	Indoor/Outdoor	Yes	No
13	Indoor/Outdoor	No	Yes
14	Indoor	No	No
15	Indoor/Outdoor	Yes	Yes

ANNEX V

Patient & otitis characteristics

Animal	Ear type	Agent	Otitis classification	Location	Otitis history
1	E	B	Erythroceruminous	Bilateral	Recurrent
2	P	F	Erythroceruminous	Bilateral	Recurrent
3	E	M	Erythroceruminous	Bilateral	First episode
4	P	M	Suppurative	Bilateral	Recurrent
5	P	M	Erythroceruminous	Unilateral – R	First episode
6	P	M	Erythroceruminous	Bilateral	Recurrent
7	P	F	Erythroceruminous	Unilateral – L	First episode
8	P	F	Erythroceruminous	Bilateral	First episode
9	P	B	Erythroceruminous	Bilateral	Recurrent
10	P	F	Erythroceruminous	Bilateral	Recurrent
11	P	F	Erythroceruminous	Bilateral	Recurrent
12	P	F	Erythroceruminous	Unilateral – R	First episode
13	P	F	Erythroceruminous	Bilateral	Recurrent
14	E	M	Erythroceruminous	Unilateral – L	First episode
15	E	B	Erythroceruminous	Bilateral	First episode

E – Erect. P – Pendulous. R – Right. L – Left. F – Fungal. B – Bacterial. M – Mixed.

This study was partially funded by Triticum®, Netherlands.